Treating the Changing Face of Western Medicine: Pharmacological Interventions on the Jak/STAT Pathway in Diabetic Complications and its Relationship to Ageing

A Thesis Presented by

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Doctor of Philosophy

I, William John Hull, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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12th May 2017

Details of collaboration and publications: I declare the assistance of George Elia of the Barts Cancer Institute, who undertook the histology in chapters III & IV and Kate Brown of Takeda Cambridge Ltd., who cut the histological samples in chapter II. Also, Julie Foster and Joseph Brook of the Barts Cancer Institute, who set up the MRI protocol in chapter IV.

Abstract

Ageing and diabetes are two major healthcare concerns that used to be regarded as problems of the Western world but are now of increasing concern in developing nations. Treating elderly patients with diabetes poses issues for clinicians due to often complex, pre-existing drug regimes. Research targeted at the development of novel drugs that have multiple effects on diabetes could go some way towards reducing polypharmacy in these patients. Here I present evidence that the oral Jak1/3 inhibitor, baricitinib, has effects on multiple aspects of diabetes. Baricitinib has been suggested to be a strong anti-inflammatory given the role Jak plays in transducing cytokine signals to elicit immune cell activation and maturation. Baricitinib was found to reduce urinary albumin to creatinine ratio and mesangial expansion in mice on an experimental high-fat diet with a diabetic metabolic profile when compared with naïve, non-diabetic mice. This reduction in renal impairment from diabetes was not found with a large reduction in proinflammatory cytokines and instead appears to be as a result of a direct effect on the cells of the mesangium. Baricitinib also reduced the circulating levels of cholesterol with a positive effect on the LDL: HDL ratio of diabetic mice. This reduction in cholesterol appears to be because of the abolition of GLP-1 signalling, initiating an increase in blood insulin, preventing lipid flux and inhibiting LDL formation. Both of these changes in key diabetic complications were *not* accompanied by an increase in sensitivity to insulin compared with vehicle treated diabetic mice. These results show that baricitinib has a beneficial effect on two key aspects of the diabetic condition but that it does not modify insulin sensitivity itself. Baricitinib may represent a potential treatment for these diabetes-associated pathologies but only in combination with traditional anti-diabetic treatments.

Contents

Publications

Throughout this programme of study, the following have been submitted:

Abstracts

W. Hull, W. Chadwick, K. Brown, S. McSweeny, D. Bedford, C. Thiemermann. Effects of DGalactose on systemic and renal inflammation: A comparison of young mice treated with DGalactose and 24-month-old mice. An oral Presentation delivered to the annual meeting of the British Pharmacological Society (December 2016).

W. Hull, W. Chadwick, K. Brown, S. McSweeny, D. Bedford, C. Thiemermann. Effects of DGalactose insulin tolerance, serum lipids and renal function: A Comparison between young mice treated with DGalactose and 24 months old mice. A poster Presentation delivered to the annual meeting of the British Pharmacological Society (December 2016).

Papers

Wayne Chadwick, William Hull, Enes Havolli, Eugene Boshoff, Mark Hill, Pascal Goetghebeur, David Harrison, Sohaib Nizami, David Bedford, Michael Bestwick, Gareth Coope, Katia Real, Helen Heffron, Joanne Doran, Johannes Grosse, Chris Thiemermann, Matt Barnes, Peter Maycox, Mark Carlton and Sarah Cole. (2017). The oDGal Mouse: A Novel, Physiologically Relevant Rodent Model of Sporadic Alzheimer's Disease. Submitted.

William Hull, Catherine Murphy, David Bedford and Chris Thiemermann. (2017). The oral Jak1/2 inhibitor baricitinib attenuates the renal damage and hypercholesterolaemia associated with diabetes mellitus in a mouse model of high-fat feeding. Submitted.

For Mum & Dad

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Abbreviations

ACE	Angiotensin-converting enzyme
ACR	Albumin to creatinine ratio
AGE	Advanced glycation end-products
AKI	Acute kidney injury
AKT	Protein kinase B
ANOVA	Analysis Of variance
Apo	Apolipoprotein
ASPA	Animals (Scientific Procedures) Act
ATP	Adenosine triphosphate
AUC	Area under the curve
cDNA	Complimentary deoxyribose nucleic acid
CKD	Chronic kidney disease
CNS	Central nervous system
CVD	Cardiovascular disease
Cyt-C	Cytochrome C
D-Gal	D-Galactose
DDM	Dodecyl maltoside
DM	Diabetes Mellitus
DMSO	Dimethyl sulphoxide
DN	Diabetic nephropathy
DNA	Deoxyribose nucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency

ESRD	End-stage renal disease
FBC	Full blood count
FDA	Food and drug administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GIP	Gastric inhibitory polypeptide
GLP1	Glucagon-like peptide-1
GLUT_	Glucose transporter
H&E	Haematoxylin and eosin
HDL	High-density lipoprotein
HFD	High fat diet
hr	Human recombinant
HSP	Heat shock protein
i.p.	Intraperitoneal
IFN	Interferon
IGF	Insulin-like growth factor
IIS	Insulin-IGF-1 system
IL-_	Interleukin-_
IRI	Ischaemia reperfusion injury
ITT	Insulin tollerance test
Jak	Janus kinase
KC	Keratinocyte chemoattractant
KIM-1	Kidney injury molecule-1
LDL	Low-density lipoprotein

LDS	Lithium dodecyl sulfate
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
mo	Months old
MRI	Magneic resonance imaging
MSD	MesoScale Discovery
NGAL	Neutrophil gelatinase-associated lipocalin
NICE	National institute for clinical excellence
OGTT	Oral glucose tollerance test
p.o.	Per os
PAS	Periodic acid Schiff
PCR	Polymerase chain reaction
PepG	Peptidoglycan
PPAR_	Peroxisome proliferator-activated receptor
PSR	Picro sirius red
q-PCR	Quantitative-polymerase chain reaction
RA	Rheumatoid arthritis
RIPA	Radioimmunoprecipitation Assay
RM	Repeated measures
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean

SPSS	Statistics package for the social sciences
SREBPS	Sterol regulatory element-binding proteins
STAT	Signal transducer and activator of transcription
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGF	Transforming groth factor
TNF	Tumor necrosis factor
UUO	Unilateral uretal obstruction
VLDL	Very-low-density lipoprotein
WBC	White blood cell

Units

C	Celsius
g	Gram
g	Gravitational acceleration
h	Hour
kDa	Kilodalton
kg	Kilogram
L	Litre
mg	Milligram
min	Minute
ml	Millilitre
nM	Nanomole
w/v	Weight/volume percentage
µg	Microgram
µl	Microlitre
wk	Weeks

Chapter I:

General Introduction & Background to Western Healthcare Issues & 'Diseases'

Ageing

The way in which medicine is practiced is constantly evolving to new healthcare challenges, among the newest changes to medicine is the way in which our western lifestyle is affecting our health. Increasing longevity in western populations has caused changes to the kinds of diseases that are causing morbidity and mortality in our societies. In order to keep ahead of the new developments related to the changing western lifestyle we must innovate and keep at the forefront of our minds the needs of the people we are trying to help. The multi-factorial nature of the task ahead means that it must be attacked on several fronts, whether that is pharmacological, societal or clinical. Another consideration as pharmacologists and clinicians is whether to treat the disease as it presents or the underlying cause of disease, so diabetes, visceral obesity or the pathology of ageing itself. Indeed, it is clear not just from now, but also from our previous experience, that if we are to truly make in-roads into this problem these approaches must be united. This thesis hopes to address one potential pharmacological option for treating diabetic complications but also look towards linking this with the pathologies of ageing which exacerbate and potentially even cause the epidemic in diabetes.

Healthcare Implications of Advancing Age

Ageing will become one of the greatest challenges to world healthcare in the coming years. Age is associated with increased hospitalisation with 23% of the global burden of disease being associated with those aged over 65 (Prince et al., 2015) despite those in this age bracket representing just 8.3% of the population (The World Bank, 2015). Cardiovascular disease (CVD) is by far the most prevalent medical issue within the over 65's (Prince et al., 2015) and this has a strong link to negative renal implications (Guerin et al., 2004).

Renal Disease in the Elderly

Renal impairment has been linked to advanced age since before advanced ageing was considered as big a health issue as it is now; Davies & Shock (1949) showed that glomerular filtration rate (GFR) declines with age. The prevalence of chronic kidney disease (CKD) in the elderly is not the only factor that is of concern. An increasing risk of death from renal disease is shown in figure 1.1 from O'Hare et al., (2007) meaning that elderly patients are not only more likely to become ill but the disease is likely to be more severe.

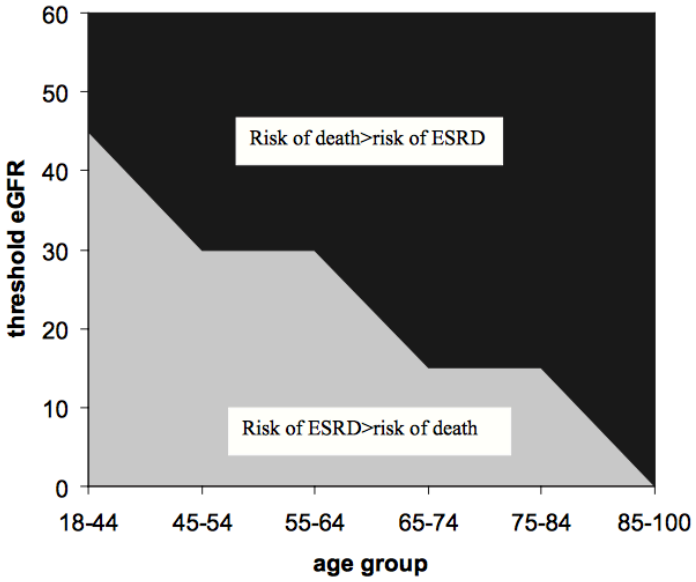

Figure 1.1. Risk of death from renal failure with age groups and GFR (from O'Hare et al., 2007).

Diabetes in the Elderly

Diabetes is a killer of the elderly, 4.6 million people die from diabetes every year with over 50% of these being over the age of 60 (Mbanya, 2012) in spite of this age group representing only around 14% of the world's diabetic population (UN, 2015). It is also known that obesity and especially chronic obesity into old age increases insulin resistance with ageing (Ahima, 2009), so ageing is known to have a contributory role in the development of diabetes.

Diabetes has long been considered the leading cause of end-stage renal disease (ESRD) in developed economies (Ghaderian et al., 2015). Its management, especially in the elderly poses a challenge to nephrologists (Abdel-Rahman et al., 2012, Joseph & Friedman, 2009) and is a leading cause of death among diabetic patients after with cardiovascular disease (CVD) (Zhu et al., 2015). Additionally, it is a great cause of morbidity and chronic pain is commonly encountered by clinicians in patients with diabetic nephropathy (DN) (Galer, Gianas & Jensen, 2000), potentially leading to further complications due to the impact of chronic pain on respiration, movement and eating (Barkin et al., 2005). These factors, combined with an increased life expectancy for diabetic patients, ageing the diabetic cohort (Miller et al., 2012), mean that diabetic complications in the elderly are of growing concern to the medical profession.

The 'Hallmarks' of Ageing

Characterisation of the 'hallmarks' of an aged phenotype is difficult to pin down to a discrete grouping of symptoms. The work of Lopez-Otin et al., in 2013 has come closest, thus far, to creating a unified theory. The Lopez-Otin et al., (2013) paper names several key hallmarks of ageing, these are changes in cellular communication, mitochondrial dysfunction, loss of nutrient sensing, deregulation of proteostasis, epigenetic changes, telomere attrition, genomic instability, cellular senescence and stem cell exhaustion. These aspects of ageing all play a role in the overall changes we see in a cell or organism. The basis of many of the ageing related investigations in this thesis relate to the hallmarks mentioned in the Lopez-Otin et al., (2013) paper framework.

Cellular communication

Cellular communication changes in ageing primarily relate to changes in inflammation signalling. The term 'inflammageing' has long been used to describe a state of chronic low-level inflammation (Franceschi et al., 2000) on ageing. Inflammageing relates to an increased cytokine profile, Salminen, Kaarniranta & Kauppinen (2012) particularly mention interleukin-1β (IL-1 β), IL-6, IL-8 and tumour necrosis factor-α (TNFα) but it would be true to say that almost all common cytokines have been noted to be elevated in advanced age. There are many causes of the up-regulated cytokine profile in ageing which have been discussed, insufficient resolution of acute inflammatory insults (Tabas, 2010) and a link to cellular senescence (Sikora, Scapagnini & Barbagallo. 2010) have both been postulated as prime causes of this observation. The downstream effects of chronic inflammation are wide ranging; Tabas (2010) notes its role in increasing CVD with age and Barzilai et al., (2012) suggests the link between inflammation in obesity and type 2 diabetes mellitus (T2DM).

This activation of the inflammation signalling network plays into the other aspects of ageing described by Lopez-Otin et al., for example 'immunosenescence'. Deeks (2011) describes immunosenescence as a case where chronic immune activation results in a weakened immune system in old age due to senescence among immune cells. Mitochondrial dysfunction may play a causative role in the activation of the inflammasome in ageing cells (Salminen et al., 2012). The role of inflammation in the pathogenesis of diabetes also links to deregulation of nutrient sensing (Hotamisligil & Erbay, 2008). Indeed, inflammation is the perfect aspect of ageing to show the truly multifactorial nature of this disease (if it can indeed be referred to as that). This is highlighted by the 2012 paper by Jenny, 'Inflammation in Aging: Cause, Effect, or Both?' this paper poses a 'chicken-and-egg' question in relation to inflammation and its role in ageing and indeed we have yet to conclusively prove the root causes of ageing.

Mitochondrial Dysfunction

As ageing progresses the efficiency and effectiveness of the mitochondrial respiratory chain decreases, this results in diminishing adenosine triphosphate (ATP) production and an increase in reactive oxygen species (ROS) from the mitochondria (Green, Galluzzi & Kroemer, 2011). Mitochondrial damage can result in the increase in permeability of the mitochondrial envelope to cytochrome-C (Cyt-C) therefore inducing apoptosome activation and cell death (Kroemer, Galluzzi & Brenner, 2007). Mitochondrial degeneration can be prevented by mitophagy and its role in clearing defective mitochondria from the cell, in turn inducing biogenesis of new mitochondria. Indeed, deregulation of mitophagy may play a role in the reduced function of aged mitochondria and thus ageing (Wang & Klionsky, 2011). The key aspect of mitochondrial failure that contributes to the ageing framework is the production of ROS, these species result in cellular damage and stress, this links to defunct proteostasis as they cause damage to proteins in the cell.

Loss of 'Proteostasis'

All cells, from a vast and complex neuron to the simplest unicellular algae rely on a network of interacting proteins to survive. These protein networks are huge; Milo (2013) estimates a range of 2–4 million proteins per cubic micron within the cell. These proteins are produced and degraded at differing rates depending on their individual characteristics (Greenbaum et al., 2003). Maintenance of the integrity of this network is vital and mechanisms for this fall into two key categories these being, protein folding and protein degradation. Incorrect or damaged proteins are often the result of a genetic mutation, however post-transcriptional damage can occur due to modifications such as oxidation or carbonylation of side chains, resulting in changes to polarity and side-chain interactions (Berlett & Stadtman, 1997, Dalle-Donne et al., 2003, Cabiscol et al., 2000).

Protein folding is controlled by the heat shock protein (HSP) family of chaperonins whereas protein degradation is mainly focused on ubiquination and the proteasome. Chaperonins play a key role in the correction of incorrectly folded proteins by 'encouraging' re-folding into a conformationally active shape, restoring function. Lower levels of protein folding correction have been shown in advancing age (Calderwood, Murshid & Prince, 2009) and failure of this system has been implicated in several ageing related diseases (Powers et al., 2009). The second branch of this system relates to proteolysis or the degradation of incorrect or damaged proteins, this is often the result of a failure of chaperonins to correct a folding error or a genetic mutation, however post-transcriptional damage by oxidation or carbonylation can induce un-correctable changes or aggregates to form (Butterfield & Kanski, 2001).

Proteolysis has two main aspects, the autophagy-lysosomal system and the ubiquitin-proteasome system. Reduced efficacies in both proteolytic systems have been implicated in ageing and ageing related diseases (Rubinsztein, Mariño & Kroemer, 2011). The ubiquitin-proteasome is shown below in figure 1.2. This system is not only used to dispose of damaged proteins but also as a regular part of proteome maintenance and regulation of signalling by degrading a signalling protein, therefore reducing signal transduction (Ley et al., 2003). The system involves the addition of ubiquitin molecules to a protein which is to be degraded, this then allows binding of the ubiquitin-conjugated protein to the 19s subunit of the proteasome. Once bound to the proteasome, the target protein enters and free amino acids and recycled ubiquitin is released.

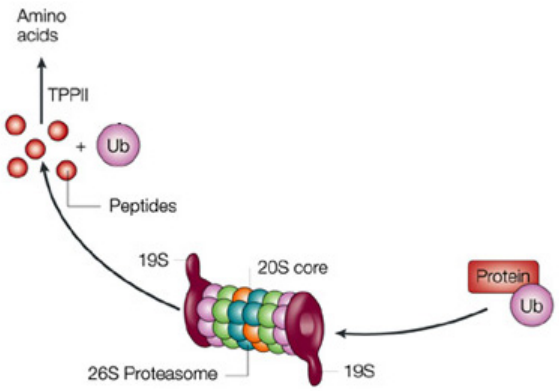

Figure 1.2. The ubiquitin-proteasome system tagging a protein for degradation allowing it to enter into the proteasome, resulting in the destruction of the protein and recycling of free amino acids (adapted from Tisdale, 2002).

The second arm of proteolysis is the autophagy-lysosomal, this system is used to degrade large amounts of protein and sometimes, whole organelles like, as seen in figure 1.3, a mitochondrion. In this process an internal vesicle (autophagosome) is formed around the item or items to be degraded, then endosomes and lysosomes fuse with the autophagosome, lowering the pH and releasing proteolytic enzymes. Failure of these proteolytic mechanisms results in the build-up of aberrant protein that may have pathology of its own or may simply no longer function, causing cellular damage. The build-up of these failed proteins can lead to diseases such as Parkinson's or Alzheimer's (Butterfield & Kanski, 2001).

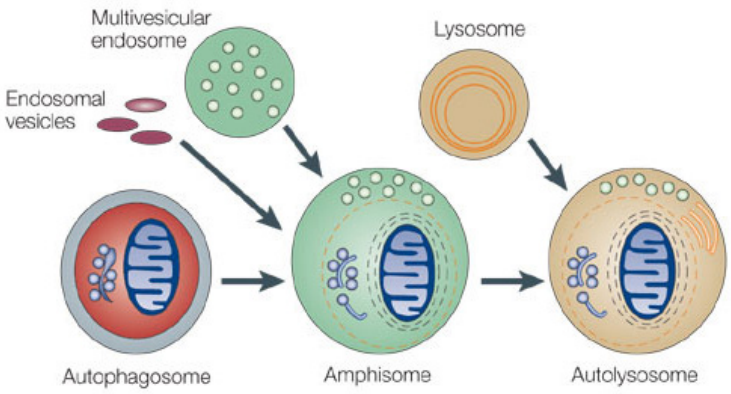

Figure 1.3. Formation of an autolysosome and for degradation of a mitochondrion (adapted from Kroemer & Jäättelä, 2005).

Loss of Nutrient Sensing

When we consider nutrient sensing in a general sense, insulin is one of the primary hormones associated with energy states. The insulin/insulin-like growth factor (IGF-1) signalling pathway (IIS) has been implicated in the activation of pathways relating to ageing and indeed knockdown and knockout models of IIS depletion have shown increased longevity (Fontana, Partridge & Longo, 2010). The practice of dietary restriction or caloric restriction has been practiced in humans with some promising results (Holloszy & Fontana, 2007) and even more promising studies in other organisms have been carried out (Panowski

et al., 2007). The studies carried out into dietary restriction have indicated that modulation of the IIS pathway is involved in the increased longevity or positive effects seen.

Insulin and IGF-1 signal through membrane bound receptors to elicit changes to cellular processes and gene transcription, this pathway is shown below in figure 1.4. The diagram of the IIS pathway shown in figure 1.4 illustrates the interplay between insulin and IGF-1 signalling. It is seen in this illustration that activation of the receptors for either insulin or IGF-1 results in a similar change to cellular energetics and transcription. Most interestingly for ageing is the ability of this pathway to induce cellular survival, proliferation and senescence via the activation of protein kinase B (AKT) or through interaction with the RAS/RAF pathway. If insulin activates pathways associated with an ageing phenotype, such as cellular senescence pathways then elevated levels are detrimental to ageing (van Heemst, 2010). Indeed, this also lends support to the idea that a reduction in caloric intake and therefore insulin increases longevity (van Heemst, 2010, Panowski et al., 2007) with this approach also linked to additional benefits like reduced oxidative stress (Sohal & Weindruch, 1996).

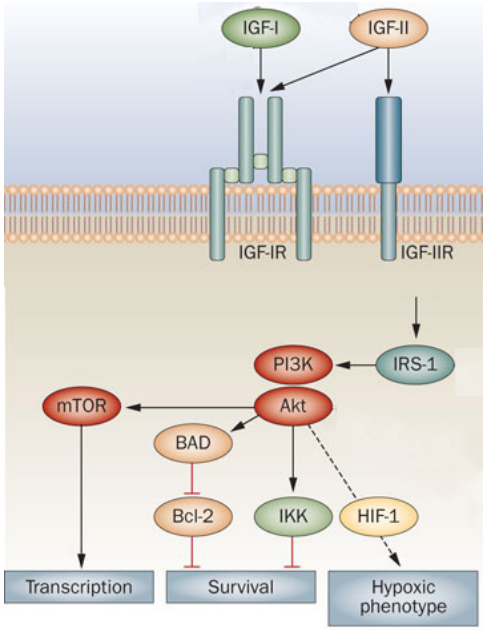

Figure 1.4. Permutations of the IIS pathway and the various outcomes of its activation (adapted from Clayton et al., 2011).

Cellular Senescence

Cellular senescence is the induction of 'stasis' in a cell whereby it is arrested in its cell cycle. Arrest in cell cycle often occurs in the G1 phase, as illustrated in figure 1.5, directly following the decondensation of chromosomal deoxyribose nucleic acids (DNA) the G1 checkpoint is reached whereby if a cell is not viable then it enters the so-called 'G0' phase. Its association with ageing has been shown by the increase in senescent cells within aged mice to ≈17% compared with ≈8% in young mice (Wang et al., 2009). Cells in senescence have been shown to have a unique combination of signals that they secrete including an increased pro-inflammatory cytokine profile (Kuilman et al., 2010). This has become known as the 'senescence-associated secretory phenotype' and may well contribute to ageing throughout an organism, given the role of cytokines in ageing, which has already been discussed.

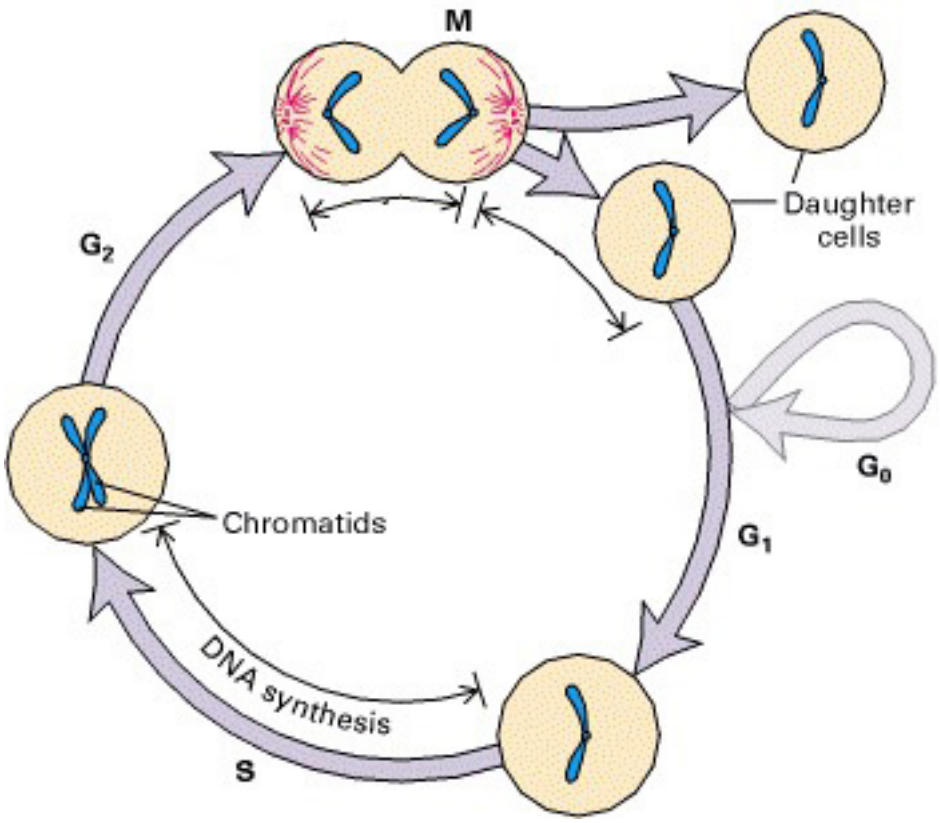

Figure 1.5. An overview of the eukaryotic cell cycle (adapted from Lodish et al., 2000).

Stem Cell Depletion

Stem cells are undifferentiated, multipotent cells capable of replacing other, terminally differentiated cells within the body. Stem cells have differing degrees of plasticity regarding the number of different cell types they can differentiate into. Depending on the cell type that needs replacing, the size of the population available to replace it may vary. The exhaustion of stem cell populations has been noted in a large number of cell populations, including the immune system with a phenomenon known as 'immunosenescence' reported by Shaw et al., (2010). The process of immunosenescence is associated with a number of changes within the aged body relating to telomere attrition, cellular senescence and inflammageing. The constitutive activation of the immune system by chronic over-expression of proinflammatory cytokines results in the exhaustion of the progenitor cells within the bone marrow. The exhaustion of these stem cell populations results in the loss of functional mass; such as muscle mass due to damaged cells no longer being replaced in old age. Reintroduction of these kinds of progenitor cells can be beneficial in repairing 'age-associated' damage and 'rejuvenating' bodies and tissues (Rando & Chang, 2012).

Genetic and Genomic Changes

Genetic and genomic changes are seen by many to be the 'root cause' of many of the hallmarks of ageing. Traditional models of biology put the genome at the centre of the cell and all processes thereof, however the delicate interplay between DNA and protein has come to the fore in terms of our thinking. Ageing associated changes to DNA include epigenetic changes (Wilson & Jones, 1983) which are only possible because of correct protein folding and genomic stability, or lack thereof which can be as a result of radical attack (Hongmei et al., 2013). Telomere attrition is the only remaining hallmark associated with the genome, this appears intrinsically linked to ageing (Armanios et al., 2009) and considering humans lack of telomerase it seems that there will be no way to avoid this hallmark.

Ultimately there are a number of ways to categorise the 'pathologies' that result from ageing. The interconnectivity of all the systems that contribute to the overall ageing phenotype mean that it cannot be boiled down to one root cause. Many systems become less effective as we age, culminating in damage on a cellular, organ and organism level.

Diabetes Mellitus

Disease Profile

Prevalence and Incidence

Diabetes Mellitus (DM) is a huge challenge for modern health systems (DH, 2001), given the advancing age and increasing rates of obesity in western populations this looks to increase within the coming years (Diabetes UK, 2017). The current statistics from the NHS show that there are around 3.6million people living with DM in the UK as of 2015 (NHS QOF, 2016). The overwhelming majority of DM cases can be divided into two main types, type 1 diabetes mellitus (T1DM), originally called insulin-dependent or juvenile onset and T2DM, originally called non-insulin-dependent. The two types differ in their aetiology and prevalence with T2DM by far being the greater health burden, representing 90% of all DM patients (Torrtora and Derrickson, 2011). Patients with T2DM have a life expectancy 10 years lower than that of the wider population (DH, 2001). There are many additional pathologies associated with T2DM often linked with vascular dysfunction and the effects of poor glycaemic control.

Clinical Presentation and Diagnosis

Patients with DM often present with lethargy, polyuria, nocturia, polydipsia, polyphagia and weight loss. Further symptoms in more advanced disease may include fainting, dizziness and sickness. Primary investigations often reveal an elevated level of blood glucose at a random blood glucose sample, further to this; fasting blood glucose test may be carried out and is usually found to be elevated also and a deranged oral glucose tolerance test (OGTT) is common. Other tests may reveal the effects of the disease on the body including proteinuria, ketoacidosis, ketonuria, peripheral numbness and visual disturbances. The gold standard diagnosis in DM is the HbA1c blood test for glycated haemaglobin that reveals levels of circulating glucose over the past 90 days (Salmasi & Dancy, 2005).

Types, Differential Diagnosis and Treatment

DM has multiple forms that each have subtly different causes, mechanisms of action and therefore treatments. The most common forms are T1DM and T2DM, however there is also gestational diabetes, a temporary decrease in insulin sensitivity in pregnant females which affects approximately 7% of pregnancies (American Diabetes Association, 2003) and diabetes maturity onset in the young, a rare genetically linked form of DM. DM should not be confused with Diabetes Insipidus which is unrelated to blood glucose levels.

Type 1 diabetes mellitus (T1DM) is characterised by depletion in the amount of insulin the body can make and therefore lower insulin levels are found in the blood stream (Heise et al., 2004). T1DM is generally accepted, to be as a result of autoimmune destruction of insulin expressing β-cells in the Islets of Langerhans of the pancreas (Yoon & Jun, 2005). The mechanism of this is well known, what is less well known is what triggers this mechanism to target these cells (Wen et al., 2005). β-cells are targeted by antibodies to glutamic acid decarboxylase 65 and islet antigen 2 among others (Borg, Fernlund & Sundkvist, 1997); this results in activation of immunity pathways and β-cell destruction.

Type 2 diabetes mellitus (T2DM) is characterised by a reduction in the body's sensitivity to the hormone insulin. Reductions in insulin sensitivity result in changes to the ability of the body to maintain glucose homeostasis (maintenance of blood glucose levels) and as a result, patients with T2DM usually have elevated blood glucose levels when uncontrolled. The mechanisms of resistance are many not always fully understood and will be explored later in this chapter. Patients with T2DM will commonly be encouraged to make lifestyle adjustments, such as losing weight. This has shown to not only reduce the risk of CVD (Espeland, 2007) but also increase hepatic insulin sensitivity (Petersen et al., 2005). The gold standard for further treatment is currently set by the national institute for clinical excellence

(NICE) in their guideline 'NG28', this is metformin, a drug used to inhibit hepatic glucose production. Other drugs recommended in NG28 include Pioglitazone, dipeptidyl peptidase-4 (DPP-4) inhibitors and glucagon-like peptide-1 (GLP-1) mimetics, which will be explored later in this chapter. Finally, it is important to note that diabetic (and particularly elderly diabetic) patients are some of the most heavily medicated patents as drugs for co-morbidities are commonly added alongside anti-diabetic drugs. T2DM differs from T1DM in that the pancreas produces sufficient insulin but this does not elicit the correct response in terms of glucose regulation where as T1DM is related to the destruction of insulin producing cells of the pancreas.

Correct differential diagnosis of DM is clearly important due to the differing treatments associated with each branch of the disease. Diagnosis of a type of diabetes is difficult and often an imperfect science as patients may present with very similar symptoms or develop one type as a result of another e.g. T2DM following gestational diabetes. BMJ Best Practice states that differentiating between T1DM and T2DM is often made on the initial presentation of the patient; they are usually under 35, not obese and may well have a family history of T1DM. Tests may include the presence of ketones in the urine, which is seldom seen in T2DM, but common in T1DM (Newton and Raskin, 2004). Additional tests would be the presence of α-GAD antibodies in the blood, which is rarely seen in T2DM (Lundgren et al., 2012). In more advanced T2DM, chronic inflammation and immune activation results in islet failure due to β-cell depletion (Donath et al., 2003), this leads to a blurring of the lines between T1DM and T2DM.

Pathology of Type 2 Diabetes Mellitus

Tight control of blood glucose is important to maintain access of the body to energy deposits and prevent damage to cells and tissues. Energy from food intakes is transported to storage or site of use in the body via the blood in the form of glucose. For the body to maintain blood glucose concentrations it must have mechanisms for both withdrawing and depositing glucose into the circulation. The mechanisms of glucose homeostasis are controlled by endocrine hormones, which are mainly released by the pancreas; the main hormones are insulin and glucagon. The pancreas has both exocrine and endocrine functions; the endocrine tissues of the pancreas are referred to as the Islets of Langerhans and contain two main types of cells, α-cells and β-cells. Deregulation of glucose homeostasis is often as a result of DM and can have long-term and short-term health effects.

Methods of Glucose Homeostasis: Insulin and Glucagon

Insulin is released from β-cells in the Islets of Langerhans of the pancreas. It is responsible for lowering blood glucose and is released in response to increased glucose concentrations in the blood (figure 1.6). During times of high blood glucose, such as just after a meal, glucose moves down its concentration gradient into β-cells via glucose transporter-2 (GLUT-2) receptors on the surface membrane. When glucose enters a β-cell it enters glycolysis and ultimately mitochondrial respiration, causing an increase in cellular ATP. Increased levels of ATP act to inhibit K^+ channels on the surface membrane of the cell causing depolarisation and therefore opening Ca^{2+} channels allowing an influx of Ca^{2+}, inducing exocytosis of vesicles containing insulin.

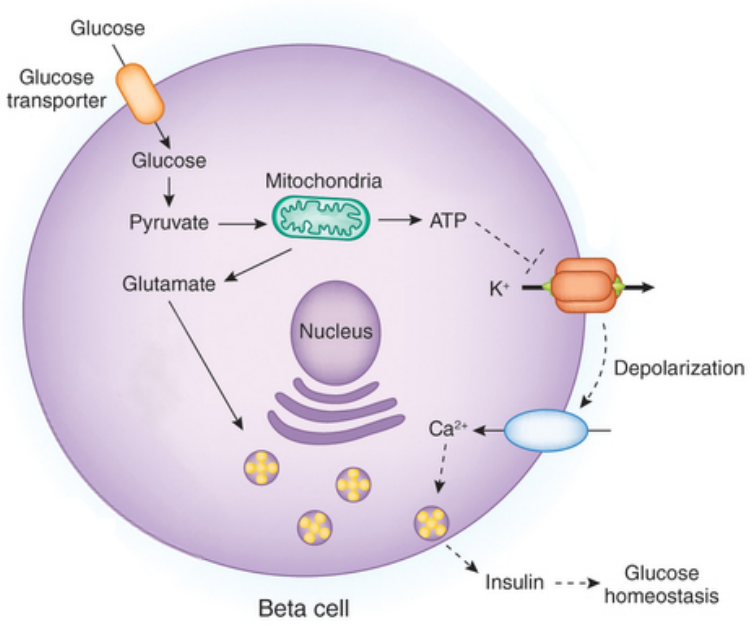

Figure 1.6. Insulin release in response to increasing blood glucose concentration (adapted from Wollheim & Maechler, 2015).

Glucagon is released in much the same fashion as insulin as depicted in figure 1.7. The difference with glucagon release is that only moderate levels of ATP will be produced due to the lower glucose availability, therefore the K^+ channels which were inhibited in the β-cells are active. This opens the T-type and L-type Ca^{2+} channels in the membrane, as with insulin, causing exocytosis of vesicles. Regulation of both insulin and glucose is also dependent on paracrine signalling from each other as well as other metabolic hormones present in the islets.

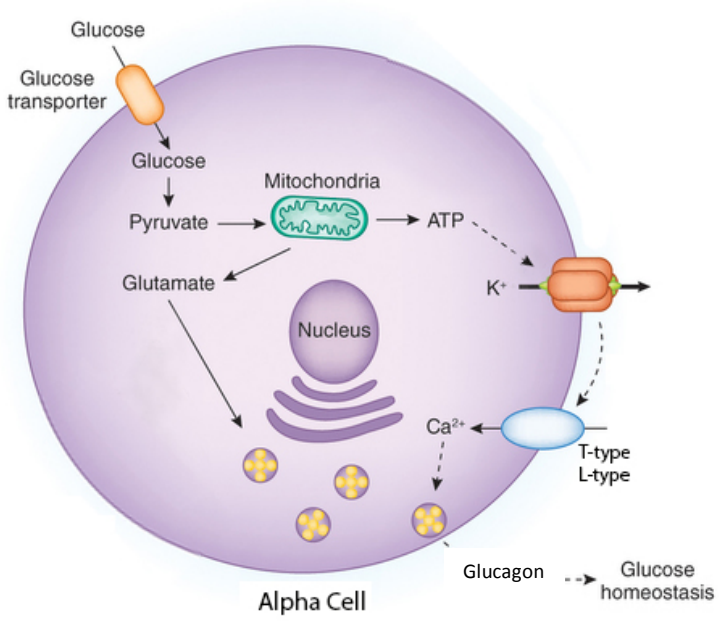

Figure 1.7. Glucagon release in response to decreasing blood glucose concentration (adapted from Wollheim & Maechler, 2015).

The hormones insulin and glucagon have effects on most cell types in the body but their main targets are muscle, fat and liver. Insulin acts to lower blood glucose, it does this by withdrawing glucose from the circulation and this takes different forms depending on the target cell. In the liver for example, insulin inhibits gluconeogenesis and glycogenolysis while promoting glycolysis and glycogenesis (Newsholme & Leech, 1983). In the muscle, glycolysis is promoted by activation of glucose-6-phosphatase (Newsholme & Leech, 1983) and increased transport of glucose into the cell is promoted by the presence of more GLUT4 glucose transporters on the surface membrane (Shepherd & Kahn, 1999). Fat has a similar

reaction to that of muscle in that it increases the number of GLUT4 transporters on its surface membrane to withdraw glucose from the blood stream (Kersten, 2001). Another effect of insulin on adipose tissue is to promote lipogensis through up regulation of a series of promoters known as sterol regulatory element–binding proteins (SREBPs) (Horton, Goldstein & Brown, 2002). In the fasting state, where there is low glucose availability, glucagon prevails and the reverse of these processes are carried out, such as hepatic gluconeogenesis, glycogenolysis and inhibition of glycolysis (plus a shift towards oxidative phosphorylation) and glycogenesis (Hüttemann et al., 2007). The mechanisms associated with the actions of glucagon serve to liberate stores of glucose, releasing it into the blood stream, resulting in an increase in blood glucose.

Aetiology of Type 2 Diabetes Mellitus

Diabetes and specifically T2DM is an interruption of the normal mechanisms of glucose homeostasis within the body, it is highly multifactorial and some facets of its pathology are poorly understood. It is essentially characterised as an inability of the body to properly sense and react to changes in insulin levels in the blood. All effects of insulin on target cells mentioned earlier in this section are depressed due to lower signal transduction. The leading theory relating to induction of insulin resistance in T2DM is the action of fat deposits, especially in key metabolic organs, the liver (Tiikkainen et al., 2004) and the muscle (Khan et al., 2015). Fat specific hormones and cytokines are believed to be key regulators and promoters of the insulin resistance associated with HFD feeding and T2DM with IL-6 and TNFα being first in consideration for this (Hotamisligil et al., 1995, Kern et al., 2001). The specific role of these cytokines in promoting insulin resistance is poorly understood (Hotamisligil et al., 1995) but it may play a role in fat remodelling (Harford et al., 2011) and this may contribute to the mechanical failures in insulin resistance. Additional roles of insulin resistance induced by fat include the inhibition of glycolysis and due to the negative feedback mechanisms of Acetyl-CoA (Randle et al., 1963), as shown in figure 1.8.

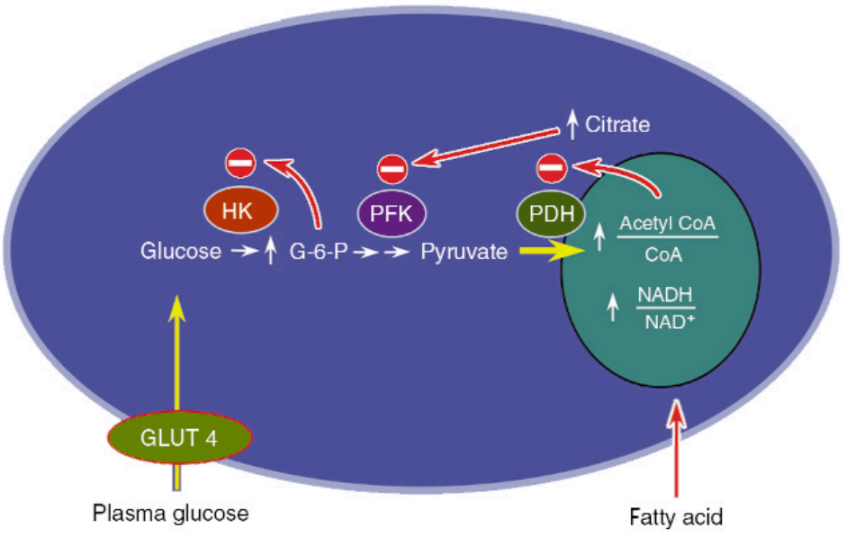

Figure 1.8. The inhibitory effects of free fatty acid availability on glucose metabolism in cells (adapted from Shulman, 2000).

Complications of Type 2 Diabetes Mellitus

Diabetes is a dangerous condition, resulting in long-term organ damage and a decreased life expectancy. Failure to control blood glucose can result in sickness, coma and ultimately death. However, few people with diabetes die as a direct result of hypo or hyperglycaemia, and by far the most common cause of death among those patients with DM is CVD (Zhu et al., 2015). The associated risk of CVD is increased with DM, resulting in a fatality rate of 52% from CVD in T2DM patients (Morrish et al., 2001). CVD has a strong link to dyslipidaemia (Pöss et al., 2011), which according to the American Diabetic Association affected 65% of diabetic patients between 2009 and 2012. The second biggest killer of diabetic patients is complications of the renal system (Zhu et al., 2015). Diabetic nephropathy (DN) is a complex renal disorder associated with renal failure and so ultimately high mortality (Ghaderian et al., 2015, Zhu et al., 2015). Indeed, DN is a risk factor for CVD and many patients will be affected by both DN and CVD (Aso, 2008). There are many other complications associated with DM, mostly relating to the circulatory and vascular system. Other complications of DM include, diabetic neuropathy, peripheral vascular disease and diabetic retinopathy, these all cause to varying degrees, morbidity and mortality of their own.

Diabetic Nephropathy

Patients with DN will usually have a past medical history of diabetes and DN is seldom the first presentation of DM due to the long-term nature of DN onset (Ewing, Campbell & Clarke, 1980). DN is often diagnosed, secondary to diabetic retinopathy as there are strong associations between the two and visual symptoms are usually easier to see in retinopathy (Lee et al., 2014). Patients will sometimes present with foamy urine, fatigue and sacral or pitting ankle oedema although the condition is commonly discovered before hypoalbuminaemia leads to these symptoms. Clinical findings include, proteinuria and hypertension with definitive diagnosis made by renal biopsy showing histological changes. Initial diagnosis is a urine albumin of 30-300 mg urinary albumin per 24 hours (Evans & Capell, 2000). This progresses over time to become more severe and being related to a decline in glomerular filtration rate (GFR). Reduced GFR is the primary measure by which renal failure is staged, mapping its progression; the use of GFR to stage renal failure is seen in table 1.1 (Levey et al., 2005).

Renal Failure Stage	Description	GFR (ml/min/1.73m^3)
1	Kidney damage with normal GFR (or sometimes mildly increased)	≥90
2	Kidney damage with mild decrease in GFR	60-89
3	Moderate decrease in GFR	30-59
4	Severe reductions in GFR	15-29
5	ESRD	<15 (dyalised)

Table 1.1. Staging of renal failure to monitor progression, using GFR.

In DN additional measures are used to track this progression the very lowest levels of microalbuminuria (between 15 and 300 µg/min) are found in stage 3. Higher levels (>0.5 g/24hr) of proteinuria are found in stage 4 where there is a rapid decline in GFR and in stage 5 where complete renal failure occurs. In ESRD it is hard to measure any renal markers as these patients will be dialysed (Mogensen, Christensen & Vittinghus, 1983). Indeed patients

in stages 1 and 2 of diabetic induced renal failure will often show no external symptoms of the disease and are missed until the disease becomes more serious, this means that early screening and additional methods to assess early stage DN are being explored (Gonzalez Suarez et al., 2013, Currie, McKay & Delles, 2014).

The major pathophysiological change in the kidney in DN is an increased permeability of the glomerular basement membrane (BM), found in the glomerulus. The Bowman's capsule surrounds the glomerulus; this is an arrangement of epithelial cells that receive the filtrated blood from the glomerulus. Blood is force filtered into the Bowman's capsule by the high pressure of afferent arteriole and the narrowing of the lumen in the efferent arteriole, meaning there is a high filtration pressure, forcing the contents of the blood into the Bowman's capsule. There is a three-tier barrier between the blood and filtrate in the Bowman's capsule; it is illustrated in figure 1.9. The first layer is the endothelial wall of the glomerular capillaries; these cells only allow filtration to happen through the gaps between them, although a little filtration occurs transcellularly, primarily of ions (Blaine et al., 2014). One of the major barriers here is the glycocalyx which is made up of glycoproteins bound to the membranes of the endothelial cells, making a filtration barrier to erythrocytes and proteins (Singh et al., 2007). The second barrier is the basement membrane, this is primarily made up of laminin and collagen (Suh & Miner. 2013) and acts to filter solutes from the blood that are smaller than 1µm or around 10kDa. The final barrier is the podocyte layer; this can be seen in figure 1.9 as the overarching cell on the glomerular side of the basement membrane. The podocytes help to maintain the integrity of the basement membrane and prevent filtration other than in the filtration slits between their 'pedicels' or 'foot-like' cytoplasmic projections. The Bowman's capsule contains the glomerular filtrate; this is rich in glucose, electrolytes and other important solutes that the body requires and so, must be absorbed.

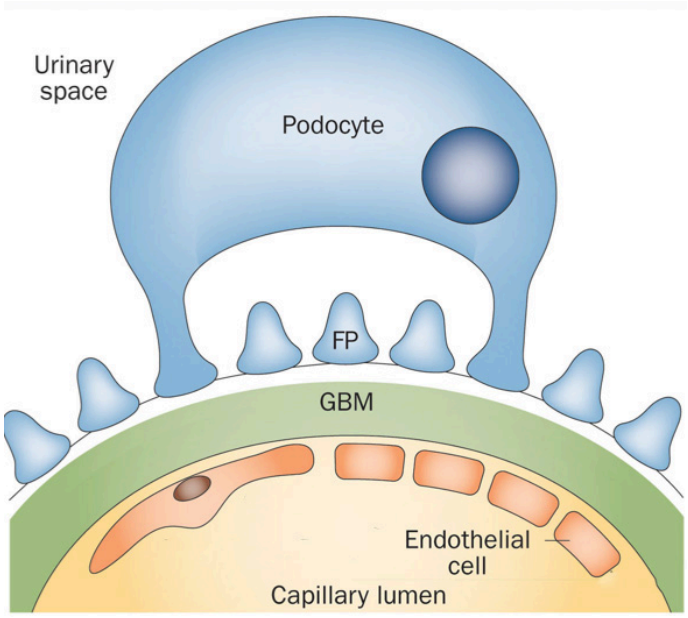

Figure 1.9. Illustration of the glomerular filtration barrier and basement membrane (adapted from Suh & Miner, 2013).

In DN there is a loss of this fine filtration structure, caused by several changes to the Bowman's capsule. Changes in the integrity of the basement membrane can be associated with several pathological changes; mechanical stresses from hypertension contribute to increases in thickness and permeability (Iversen & Ofstad, 1987). Inflammation results in changes in the cells of the Bowman's capsule including vascular endothelial (Feener & King, 1996), mesangial (Steffes et al., 1989) and podocyte (Susztak et al., 2006) cells while also attracting immune cells to migrate into the kidney (Williams & Davies, 1987), though the exact role of inflammation in BM thickening is poorly understood (Dalla Vestra et al., 2005).

Mesangial expansion is known to have a big part to play in the pathogenesis of DN. Mesangial cells help to maintain the shape of the 'tuft of blood vessels in the glomerulus and are situated at the 'neck of this', their location is shown in figure 1.10. Expansion of the mesangium and mesangial matrix is central to the development and progression of DN (Mauer et al., 1984). Mesangial expansion is linked to the occlusion of the glomerular blood vessels, leading to the death of podocytes and eventual collapse of the glomerular apparatus (Østerby et al., 1990, Harris R et al., 1991).

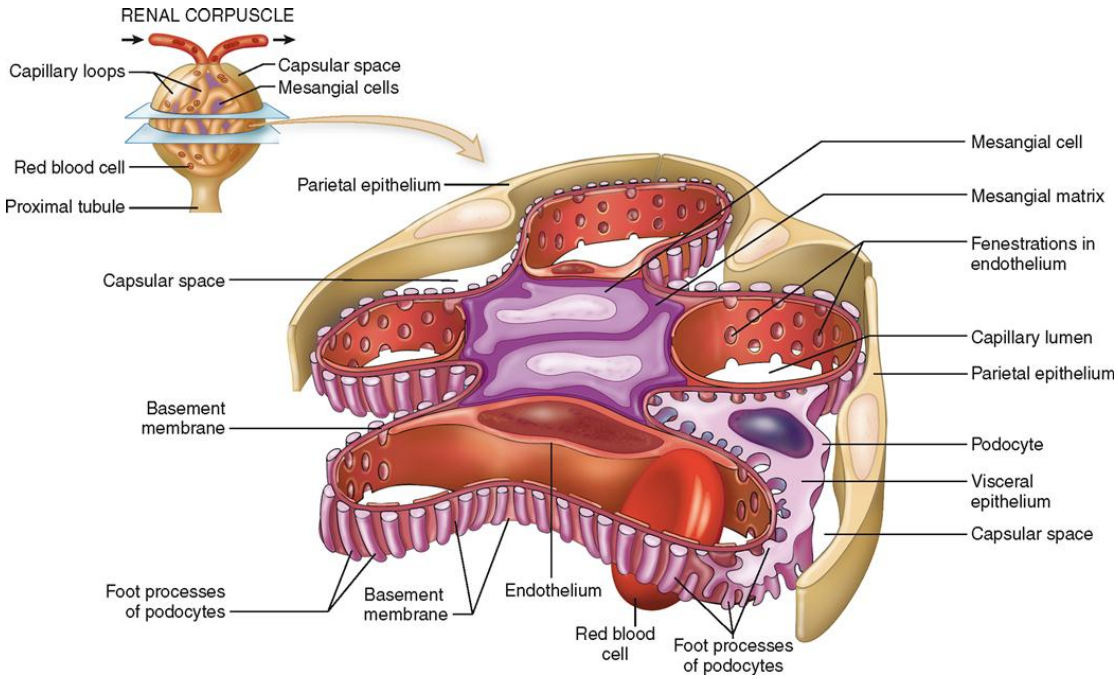

Figure 1.10. A cross-sectional illustration of the glomerular apparatus (from Basicmedical Key, 2016).

Glucose control is also considered a major contributing factor to BM thickening and loss of integrity (Evans & Capell, 2000). Advanced glycation end-products (AGE) are created by the non-enzymatic addition of glucose molecules to proteins in the body, a process unregulated in the case of DM due to chronic higher levels of glucose (Ahmed, 2005). When proteins become glycated this often changes their ability to interact with other proteins and with cells. A change in the conformation of a protein of the BM or glycocalyx would result in the interactions it makes in order to physically form a filtration barrier to break down, causing

filtration to fail (Forbes et al., 2003). The multiplicity of factors that cause DN in the glomerulus mean that there are potentially many targets for pharmacological intervention but that is a double-edged sword in that we do not yet have one uniting cause to target.

Diabetic Dyslipidaemia

Failure of insulin signalling in T2DM results in characteristic changes to serum lipid profiles, increases triglyceride, free fatty acid (FFA), low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL) and a decrease in high-density lipoprotein (HDL) (Goldberg, 2001). This kind of lipid profile in a patient suggests a high risk of CVD (Howard et al., 2000). The national institute for clinical excellence (NICE) currently recommends high intensity statin treatment for patients with diabetes to help control risk of CVD (NICE, 2016). High intensity statin treatment means Simvastatin 80 mg, this is however controversial given studies that show Simvastatin has an increased risk of myopathy (MHRA, 2010).

Dyslipidaemia in T2DM appears to be due to an increased so-called 'fatty acid flux', this process was first described by Randle et al., in 1963. This is the process whereby fatty acids are liberated from the triglyceride rich stores of adipose tissue and returned to the liver to be reprocessed into HDL, LDL etc. The cycle is a complex one, involving many hormones and regulatory proteins, an overview of this process can be seen in figure 1.11. Following stimulation from a number of signals, such as increasing insulin resistance and cytokines, adipocytes release FFA due to stimulation of lipoprotein lipase (LPL). These enter the circulation and are withdrawn from the circulation by hepatocytes, which express apolipoproteins (Apo). Apo is usually broken down when produced unless bound to fat, the increased presence of fat causes these proteins to bind to fat and become lipoproteins, like LDL and VLDL which are produced preferentially to HDL in the presence of high fat (Goldberg, 2001).

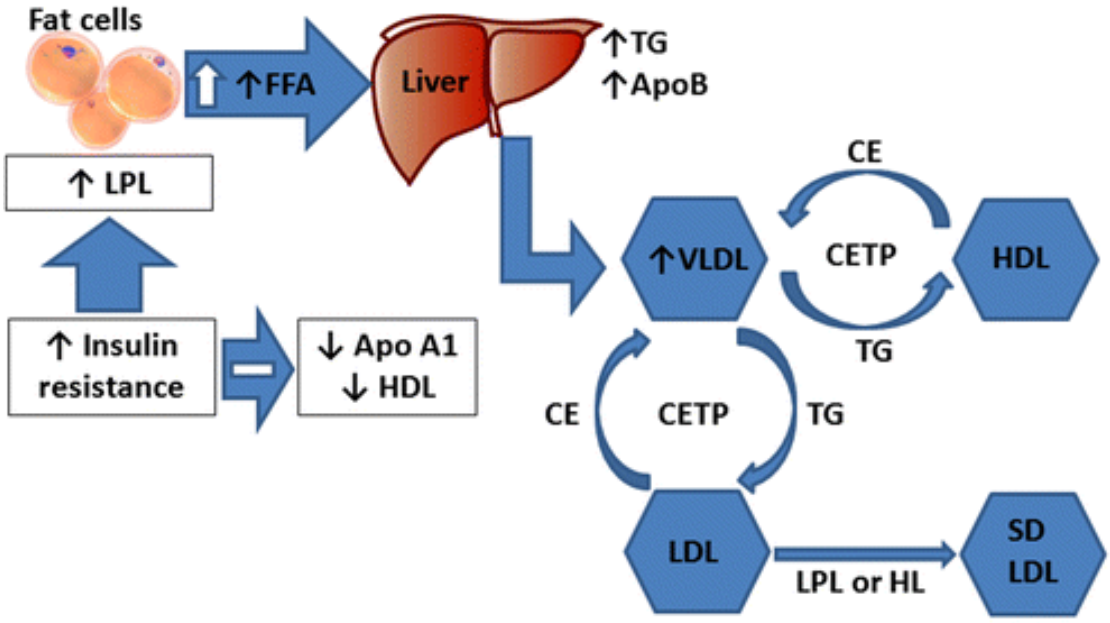

Figure 1.11. Pathogenesis of diabetic dyslipidaemia. Apo A-1 apolipoprotein A-1, Apo B apolipoprotein B, CE cholesteryl ester, CETP cholesteryl ester transfer protein, FFA free fatty acid, HDL high density lipoprotein, HL hepatic lipase, LDL low density lipoprotein, LPL lipoprotein lipase, SD LDL small dense LDL cholesterol, TG triglyceride, TNF-α tumour necrosis factor α, VLDL very low density lipoprotein (adapted from Chehade, Gladysz & Mooradian, 2013).

The Jak/STAT Signalling Pathway

Jak/STAT as a Common Signal Transduction Pathway

The Jak/STAT pathway acts as part of cytokine signalling pathways, it is associated with signal transduction from cellular membrane receptors to the nucleus. The Janus kinase (Jak) family were discovered as part of a wide screen of kinases performed by Wilkes (1989). These cytosolic proteins were found to be non-receptor kinases that associated with signal transducer and activator of transcription (STAT) proteins resulting in the phosphorylation and activation of these cytosolic proteins. The second part of the Jak/STAT pathway is the STAT family of proteins. STATs are transcription factors that, when activated, translocate to the nucleus of a cell and initiate gene transcription.

Pathway Summary

The Jak/STAT pathway in its simplest form is described in figure 1.12. Figure 1.12 shows the dimerisation of a transmembrane receptor, both sub units of which associate with a Jak (in this case Jak1 and TYK2). After dimerisation the Jaks then phosphorylate each other activating their kinase domains, resulting in the phosphorylation of tyrosine residues on the cytokine receptors intercellular domains. Once fully activated, the Jak/receptor complex can bind STAT transducer proteins that are phosphorylated in order to activate them. Activated STAT proteins dimerise forming hetero or homodimers (Williams, 2000) it is only when STATS associate with one another they can translocate to the nucleus to act as transcription factors.

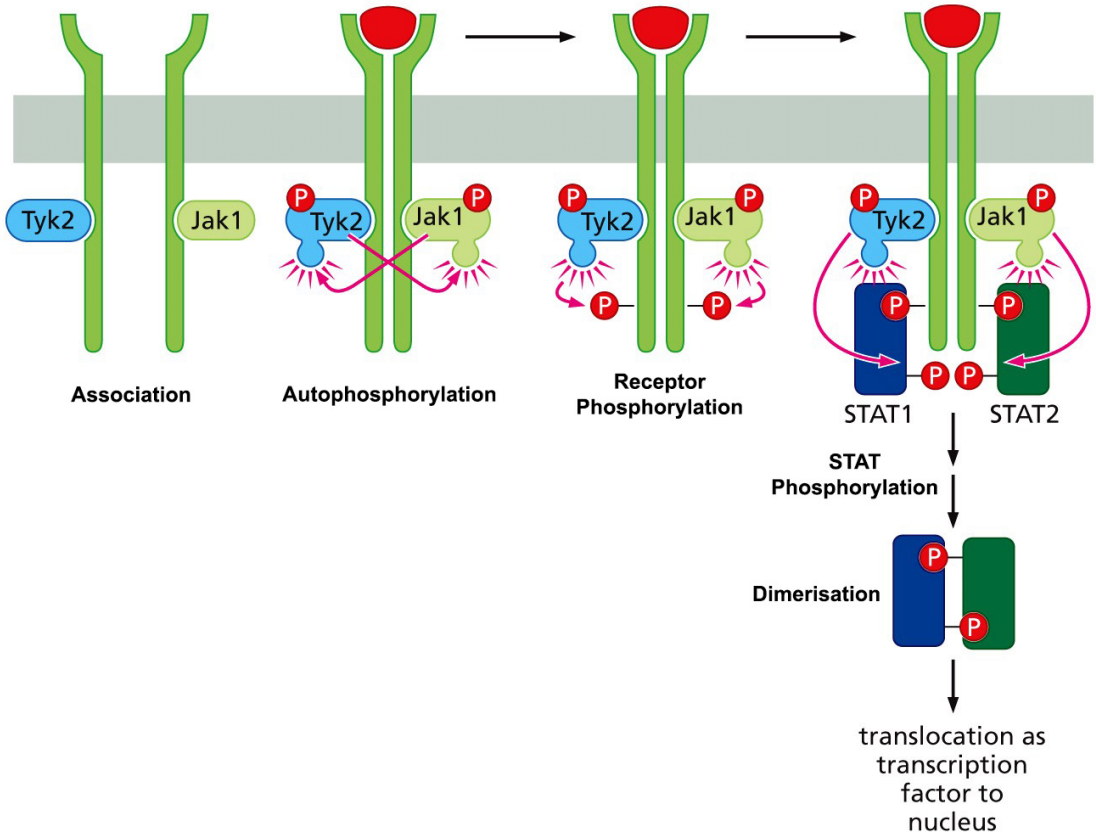

Figure 1.12. Jak/STAT pathway activation (from Weinberg, 2013).

As with all biology, the Jak/STAT pathway is far more complex than has been indicated here in figure 1.12. The multiplicity of receptors that associate with Jaks, of which there are 4 and then 7 STATs that can all form dimers with each other means that there is a large web of signalling rather than a straightforward signalling pathway. The total number of combinations that can be formed with activation of this pathway is 784, showing the huge redundancy in this system. Although is it true that the Jaks do not all associate with the same receptors for cytokines, figure 1.13 shows which Jaks preferentially associate with which cytokine receptors. The map in figure 1.13 is by no means exhaustive but gives an idea of the kind of receptors that link to the Jak/STAT pathway (Murray, 2007).

	Jak1	Jak2	Jak3	Tyk2
Jak1	IL-10, IL-6, IL-11, IL-22			
Jak2	IFNγ	IL-3, IL-5		
Jak3	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21			
Tyk2	IFNα/β	IL-12, IL-23		

Figure 1.13. Map of Jak association with receptors for cytokine signalling.

In figure 1.14 is illustrated the kinds of cytokines and how their receptors preferentially associate with different Jaks, resulting in different STAT combinations, different gene expression profiles and therefore different downstream effects.

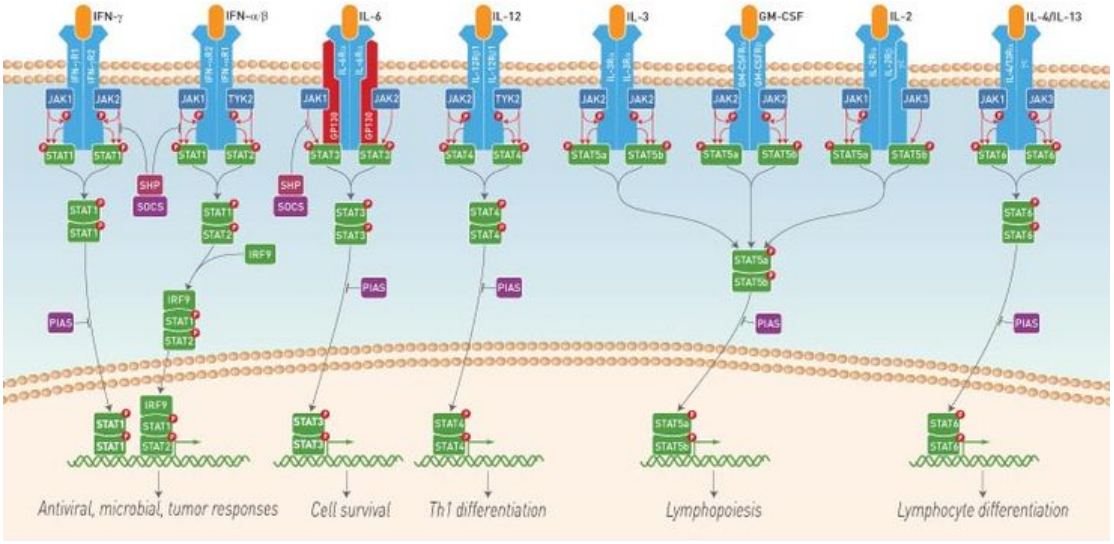

Figure 1.14. Various cytokine/receptor-Jak/STAT-gene interactions (adapted from lifetechnologies.com/jakstat).

Janus Kinases

The Jak family of non-receptor protein tyrosine kinases has 4 members, these being Jak1, Jak2, Tyk2 and Jak3. These proteins associate with the cytosolic domains of activated cytokine receptor dimers, resulting in the phosphorylation of key tyrosine residues in the 'inactivation loop' (Schindler et al., 2007). The phosphorylation of Jaks is mediated both by the association of Jak with a receptor and the action of the other Jak bound to the alternative receptor subunit as both Jaks are responsible for phosphorylation of each other. Jaks exclusively associate with STAT proteins and Jak activation will always result in the activation of STATs by phosphorylation, STATs however are more promiscuous and can be activated by other kinases.

Signal Transducers and Activators of Transcription

The STATs are a 7-member family of transcription factors, containing STATs 1-4, 5a and 5b as well as STAT6. All stats contain a conserved tyrosine activation domain where the activating tyrosine residue becomes phosphorylated (Schindler et al., 2007). Another key domain of the STAT protein in is the DNA Binding Domain (DBD) that allows for association with enhancer DNA sequences. Upon activation by phosphorylation, STAT proteins associate with one another to form either hetero or homo-dimers that subsequently translocate to the nucleus to enable them to bind to DNA enhancer sequences (Jatiani et al., 2010).

Suppressors of Cytokine Signalling

Genes regulated downstream of Jak/STAT activation are varied in their function depending on the cell type upon which the cytokine is acting and which STATs are phosphorylated. One family of genes that are important in all Jak/STAT signalling are the suppressors of cytokine signalling (SOCS). These act as a negative feedback and regulator on the Jak/STAT pathway (Croker et al., 2008).

Common functions and Examples

A simple gene atlas search for the machinery of the Jak/STAT pathway will show it is expressed at some level in almost all tissues but most highly in immune cells of the lymphoid lineage. This tissue specific expression is due to the machinery of the pathway and their role in cytokine signal transduction. The Jak/STAT pathway plays a key part in lymphopoesis and the maturation of immune cells. Figure 1.15 shows a T-Cell being activated by an activated B-Cell, in this case, both cells are being activated via the Jak/STAT pathway as both IL-2 (Bright, Kerr & Sriram, 1997) and IL-4 (Jiang H, Harris M & Rothman P, 2000) receptors associate with Jaks to transduce their signal evoke changes in the cell. In figure 1.17 the T-Cell is self-activating via the expression of IL-2 and is also further activating the B-Cell by expressing IL-4, this cell would a T-helper (T_h) cell. T_h-cells could be considered the 'cytokine

factories' of the body. T_h-Cells are responsible for the activation of the humeral immune response, neutrophils, macrophages, fibroblasts and T_c-Cells. T_h-Cells fall into two main categories, being Type-1 (Th1) and Type-2 (Th2) (Carter & Dutton, 1996). The primary activator for both Th and Tc cells is IL-2; this is released by T_h-Cells and activates all T-Cell types in an autocrine fashion. IL-2 binds to IL-2 receptors expressed on the surface of T-Cells as well as dendritic cells and other immune targets (Boyman & Sprent, 2012) inducing activation in a positive feedback loop. Another key cytokine produced by Th1 cells is Interferon-γ (IFNγ); this activates macrophages, the main target of Th1 cells (Mosser & Edwards, 2008). Tc-Cells express IL-2 receptors on their surface and as such are susceptible to IL-2 mediated activation that can be from T_h-Cells or even IL-2 generated by their self-activation when they bind to an antigen. Th2 cells play a role in the activation of B-Cells and so the activation of the humeral immune response.

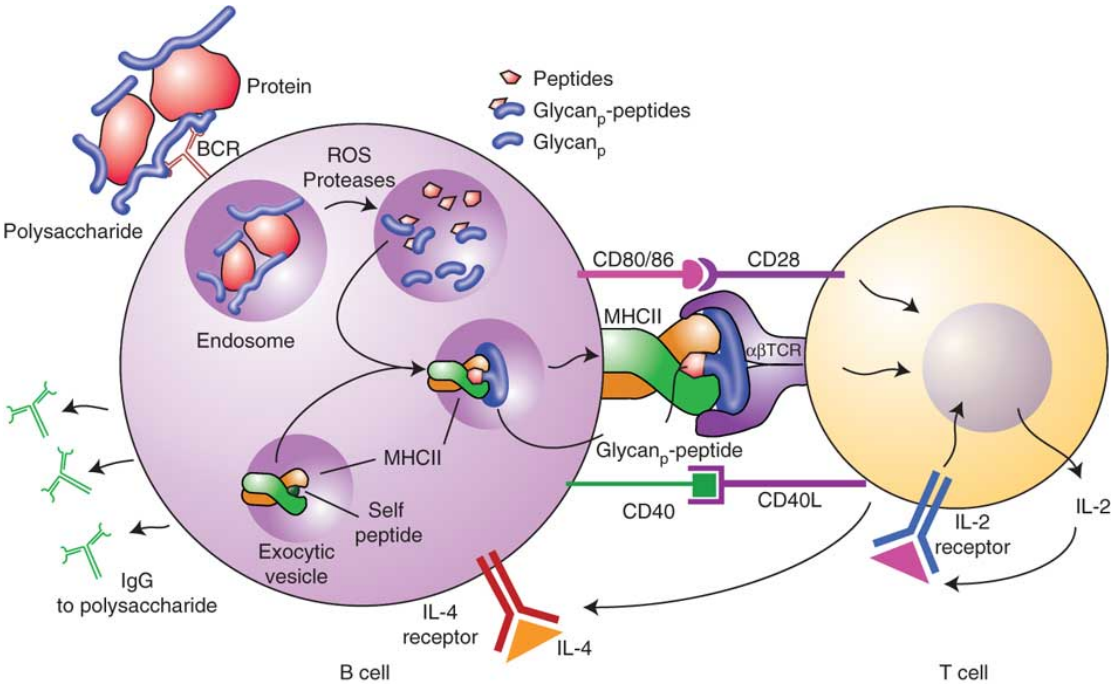

Figure 1.15. B-Cell and T-Cell interactions resulting in activation of both cells via cytokine signalling through the Jak/STAT pathway (adapted from Avci et al., 2011).

The Jak/STAT Pathway as a Target For Pharmacological Intervention

The mechanism of Jak inhibitor efficacy is poorly understood and there is debate as to the role of Jak inhibition in disease states. Okinaga et al., (2012) suggests a role for STAT3 phosphorylation in activation of $p21^{cip1/waf1}$ and induction of cell cycle arrest and apoptosis. This role as an anti-apoptotic is mirrored in a lot of the cancer related literature (Quelle et al., 1998, Rajasingh et al., 2006) on the subject of Jak/STAT signalling in cell death pathway activation. The other side of the Jakinib story is the idea that inhibitors are general anti-inflammatories; certainly there is a strong inflammatory component of in RA, for which Jakinibs have been used, leading credence to this idea. There are a number of papers published on the inhibition of Jaks being anti-inflammatory (Kudlacz et al., 2008) or Jaks playing a role in strongly inflammatory diseases (Gyurkovska & Ivanovska, 2015). Inhibition of the Jaks is not uncommon and there are many so-called 'Jakinibs' in early clinical usage as well as others in clinical trial phases. The primary aim of these drugs is in Rheumatoid Arthritis (RA) as the Jak pathway is involved in activation of T-cells.

Jak/STAT in Renal Disease

The Jak/STAT pathway has been suggested as a potential target for kidney disease (Chuang & He, 2010) but there is as yet, little published evidence of studies into the effects of inhibition on the severity of kidney disease. That which has been published or is currently being investigated has focused on diabetic kidney disease (DN) (Berthier et al., 2009). A phase II clinical trial recently concluded in the USA that used a Jak 1/2 inhibitor called baricitinib (Eli Lilly) in DN patients and presented its findings at the 2015 meeting of the American diabetes association. This Phase two trial showed a dose dependent reduction in the urine albumin-creatinine ratio (ACR) for patients treated with baricitinib over placebo. This makes the inhibition of this pathway and usage of these Jakinibs potentially interesting in other progressive kidney diseases.

Pathway inhibition is postulated to be beneficial in several renal pathologies, not just confined to a specific type of injury (Chuang & He, 2010). Unilateral uretal obstruction (UUO) models were used to evaluate the role of STAT3 signalling on renal fibrosis with positive results (Pang et al., 2010, Kuratsune et al., 2007). HIV associated nephropathy (Cijiang He et al., 2004) and anti-Thy 1.1 induced nephropathy (Yanagita et al., 2001) models have been shown to have an element of Jak/STAT signalling in their pathology. Finally, ischaemia/reperfusion induced acute kidney injury (AKI) has been postulated to have as a potential target the Jak/STAT pathway and experimental blockage has yielded potentially interesting results (Li et al., 2007, Yang et al., 2008). These results are encouraging as to the potential for Jakinibs in renal diseases of all sorts, not just glomerulopathies but also tubular injury and fibrosis.

Aims of Investigations Presented in this Thesis

- Assess the feasibility of using an animal model of 'artificial ageing' in nephrology research

- Characterise key areas of natural renal aging for comparison to diseases with an 'ageing-like phenotype'

- Establish modifications associated with Jak/STAT activation in ageing

- Extrapolate Jak/STAT activation analysis to diseases with an 'ageing-like phenotype'

- Use the oral Jak1/2 inhibitor 'baricitinib' in an animal model of diabetic nephropathy

- Explore the cholesterol lowering mode of action of the oral Jak1/2 inhibitor 'baricitinib'

- Unite knowledge of the Jak/STAT pathway in ageing and diabetic nephropathy to make links that could benefit tailored treatment of elderly patients with these types of drugs

Chapter II: Ageing and the D-Galactose Model

Introduction

It was reported in the 1990's that low dose D-Galactose (DGal) administered to mice could induce them to display an 'ageing like phenotype' (Song et al., 1999). Throughout the 1990's and 2000's this model become increasingly popular in China especially as a means of creating an ageing 'disease model' *in vivo* (Ho et al., 2003, Wei et al., 2005). The development of this model was focused on its impact on the central nervous system (CNS) and induction of behavioural changes (Wei et al., 2005). Studies attempting to elucidate the mechanism by which these changes in behaviour were induced concluded that DGal changed the state of the redox environment, inducing oxidative stress in the brain (Cui et al., 2006), resulting in increased AGE (Ho et al., 2003). Another observation made with this model was that neuro-inflammation was increased in animals subjected to DGal treatment and that this was as a result of the oxidative stress (Lu et al., 2010).

Ageing has for a long time been considered at least in part, a result of inappropriate chronic inflammation (Licastro et al., 2005). This chronic inflammation is suspected to be linked to ROS and other radical species generated as part of normal inflammation and metabolism (Raha & Robinson, 2000, Finkel & Holbrook, 2000). Ageing in the kidney is often considered to be accelerated in the diseased state (Giachelli, 2004, Ferenbach & Bonventre. 2015). The link between ROS, inflammation and ageing leads to the idea that, should DGal indeed act via a mechanism of oxidative stress, it could be used to create a mimetic model of ageing in all organs, not just the brain.

Ageing is an extremely complex multifactorial process, which especially in humans varies greatly from person to person in its perceived rate of advance. An increasing ageing population, like that which we see in the UK and other developed nations, will be associated

with an increased morbidity and therefore, stress on healthcare systems (Christensen et al., 2009). Developed nations are already spending almost four times more on healthcare for the elderly than for the remaining population (Anderson & Hussey, 2000).

Renal impairment increases with advancing age and certainly will form a part of the increased burden on healthcare systems in the coming years (Stevens et al., 2010). Renal disease is a risk factor for the development of CVD (Foley et al., 2005) with some studies putting the risk of patients with chronic kidney disease (CKD) dying from cardiovascular complications at twice that of non-CKD patients (Collins et al., 2003, Go et al., 2004). This shows that in the future, with an ageing population, renal insufficiency and renal disease will need to be addressed as a cause of morbidity and mortality in the UK and around the world. It is fair to say that there is a need for development of novel treatments in all aspects of renal disease taking into account the differing needs of an older renal patient.

Some evidence of an increasingly oxidative environment outside the CNS has been published (Aydin et al., 2012) suggesting that the kidney is also susceptible to the DGal 'model of ageing'. It would seem sensible in light of this knowledge and the need for treatments for older patients to apply a DGal model to renal research.

The proposal of this investigation was to begin evaluating the impact of DGal administration on the laboratory mouse outside of the CNS to look at it from a holistic standpoint. Many factors such as metabolic ability and specifically renal function were to be measured in mice administered DGal versus naturally aged mice. The hope of this investigation was to establish whether the DGal model was truly mimetic of ageing or if it was a more specific CNS related effect.

The model we used differed from that used in all the previously published literature in that DGal was to be administered in the drinking water rather than by intraperitoneal (i.p.) or subcutaneously (s.c.) injection. Due to the Home Office guidance and relating legislation under the animals (scientific procedures) act (ASPA) it is not possible to chronically inject DGal for 6-8wk. This model had previously been proved to induce cognitive deficit and increased brain inflammation in mice in-house at Takeda Cambridge Ltd. (Chadwick et al., 2017, under review).

Materials and Methods

In vivo

All *in vivo* experiments were carried out on C57BL/6J mice obtained from Charles River UK Ltd. (Margate, Kent) and each of the below described procedures were carried out on the same individuals within all cohorts unless otherwise specified. Mice were received into the biological services unit at Takeda Cambridge Ltd. at 9wk of age and housed under home office regulations until sacrificed at 3 (3mo), 12 (12mo) or 24 (24mo) months of age. Mice in DGal treated groups were housed in the same manner as 3mo mice and received DGal in their drinking water at doses of 2g/kg/day (low dose), 4g/kg/day (high dose) or vehicle (0.1% w/v sodium benzoate) for 6wk prior to commencement of studies. Dosages of DGal were based on earlier data that suggested mice under these conditions drink approximately 2ml per day.

MRI for Body Composition

Mice were removed from cages, weighed, placed into retainers for a period of approximately 5min while the MRI machine (EchoMRI™ LLC, Houston, Texas, USA) was operational and then returned to cages following the completion of measurements, with body composition calculated by the MRI machine software from EchoMRI™ LLC (Houston, Texas, USA).

Fast/Re-Feed Food Intake

Mice were subjected to a 16hr overnight fast the day preceding the study by moving them into a new clean cage without food but water *ad libitum*. Following fasting, food was re-introduced into the cages at a pre-determined weight. The food intake of whole cages was then measured by weighing the food at 1, 2, 4, 8 and 24h post re-introduction of food, this data was then averaged for the number of mice in each cage.

Measurement of Blood Glucose

At numerous points throughout the study it was necessary to measure the blood glucose levels of the mice. Blood glucose measurement was carried out by removing a very small 1mm section of the end of the tail with scissors allowing collection of a small drop of blood. Blood removed from the tail was tested using a glucosemeter (OneTouch®-Ultra, LifeScan) with glucose test strips (Accu-Chek®, Roche).

Full Blood Count (FBC)

Blood samples for the full blood counts were collected in the same way as earlier described for measurement of blood glucose, using a tail bleed. Approximately 50µl of blood was collected into a capillary tube and 20µl used for automated analysis in the ABC Vet Automated Blood Counter 16p (Woodley Equipment Company Ltd., Bolton UK).

Oral Glucose Tolerance Test (OGTT)

Mice were fasted overnight (16h) by transferring to a new clean cage with no access to food but water *ad libitum*. Before beginning protocol the weight of each mouse was established and volume of 15% w/v glucose solution required for a dose of 1.5mg/g calculated. Fasted blood glucose and blood insulin (determined later by enzyme-linked immunosorbent assay (ELISA)) were measured, from tail-vein blood, before glucose was administered by oral gavage. Blood glucose was measured at T=10min, 30min, 45min, 60min and 90min with insulin also being measured at T=10min and T=45min.

Insulin Tolerance Test (ITT)

Fasting for 4h was performed to normalise insulin levels prior to initiation of the test. Mice were weighed and a volume of insulin for a dose of 0.75U/kg established. Baseline blood glucose measurements were then made and the insulin administered i.p. Blood glucose measurements were made from tail-vein blood at 15, 30, 45, 60, 90 and 120min post administration of insulin.

Sacrifice and Terminal Dissection

All animals were killed by the approved schedule 1 method of increasing concentration of CO_2 until dead; conformation of death was exsanguination by cardiac puncture. Blood collected form cardiac puncture was then spun at 3000g for 5min, serum was collected and stored at -80°C in aliquots for later analysis. Mice were fasted overnight (16h) prior to sacrifice. Mice were immediately dissected after cardiac puncture and all organs stored in 10% formalin, RNALater® or at -80°C for later analysis.

Ex vivo

All *ex vivo* experiments were carried out on samples gathered from the animals described in the *in vivo* section.

Serum Biochemistry

Serum biochemistry was carried out by Dr Keith Burling of the Core Biochemical Assay Laboratory (Cambridge, UK) using the Siemens Dimension EXL autoanalyser.

Insulin ELISA

Insulin ELISA was carried out using commercially available insulin ELISA kit (Abcam, Cambridge, UK) on whole blood collected from the tail vein of mice during OGTT following protocol set out by the manufacturer.

Urine Renal Biomarker ELISAs

Urine biomarker assays were carried out on urine collected by housing mice in metabolic cages for a period of 24h. ELISAs were run for albumin (Abcam. Cambridge, UK), lipocan-1 (NGAL) (R&D Systems. Minneapolis, MN, USA), kidney injury molecule-1 (Kim-1) (Aviscera Sciences. Santa Clara, CA, USA) and creatinine (Crystal Chem. Downers Grove, IL, USA) using commercially available kits and following manufacturers protocols.

Tissue Lysis Protocol

All tissues were lysed in 1% ice-cold dodecyl maltoside (DDM) lysis buffer using QIAshredder™ (Qiagen, Hilden, Germany) tissue homogeniser. Tissues were weighed and 1:5 w/v of ice-cold 1% DDM buffer added before homogenisation. After homogenisation, lysates were centrifuged at 3000g for 5min and supernatant collected with both supernatant and pellet stored at -80°C for later analysis.

Cytokine Analysis

Cytokine levels in tissue and serum were analysed using commercially available, multiplex (Mouse ProInflammatory 7-Plex) assay kits from MesoScale Diagnostics (MSD) LLC. (Rockville, MD, USA) following the protocol set down by the manufacturer. Assays were carried out on supernatant of lysate and serum collected at terminal dissection.

Advanced Glycation End-Products (AGE) and Protein Carbonyl ELISAs

ELISAs for both AGE and protein carbonyl were performed on tissue lyste supernatant and re-solublised pellet. Both ELISAs were commercially available kits from Cell Biolabs Inc. (San Diego, CA, USA)

Histological Analysis

Histological analysis was made on 4μm samples of tissue that had been fixed in 10% formalin for 24h and embedded in paraffin. Slides were deparaffinised in xylene, rehydrated and stained with Mayers's haematoxylin and eosin (H&E) and dehydrated before having a coverslip applied with distyrene, a plasticizer and xylene (DPX) mountant. These slides were viewed using a Nanozoomer digital pathology scanner (Hamamatsu Photonics K.K., Japan). Glomerular and Bowman's capsule sizes were assessed using the Nanozoomer NDP viewer software (Hamamatsu Photonics K.K., Japan). Two cross-sectional measurements (at 90° to one another) were taken for 20 renal corpuscles and averaged before an area for each was established using the formula $A=\pi r^2$.

RNA Extraction

Samples of organs stored in RNALater® had ribose nucleic acids (RNA) extracted using commercially available RNEasy spin columns from Qaigen (Hilden, Germany) following their procedure with the RNA stored at -80°C for later analysis.

cDNA Synthesis

cDNA synthesis for analysis by quantitative polymerase chain-reaction (q-PCR) was carried out using QuantiTect® reverse transcription kit from Qiagen (Hilden, Germany) following instructions laid out by the manufacturer. The cDNA was stored at -20°C for later analysis with excessive freeze-thaw-cycles avoided.

Quantitive-PCR (q-PCR)

cDNA samples synthesised from RNA samples from mouse organs were tested in q-PCR reactions. These reactions were set up in 96-well plates using TaqMan® Fast universal PCR master mix (ThermoFisher, MA, USA). Probes were VIC-labelled, MGB-quenched glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe for control housekeeping gene and FAM-labelled, MGB-quenched gene of interest probes. Protocol was run using One Step Plus q-PCR machine from Applied Biosystems and data returned in the form of raw C_t values that were internally normalised twice against GAPDH and control sample to produce $2\Delta\Delta C_t$ values as presented.

Materials

All reagents were acquired from Sigma-Adrich Ltd. (Milan, Italy) unless otherwise stated.

Statistical Analysis

All values described are presented as mean ± standard error of the mean (SEM) for n replicates. Statistical validation and exclusion testing was performed using the statistics package for the social sciences (SPSS) from IBM (Armonk, NY, USA). Area under the curve (AUC) analysis was carried out using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA). One-way analysis of variance (ANOVA) with Bonferroni's post-hoc test was carried out on data sets using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA) and a *P*-value of ≤0.05 was considered to be significant. Also used was two-way repeated measures (RM) ANOVA with Bonferroni's post-hoc test using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA) and a *P*-value of ≤0.05 was considered to be significant.

Results

Metabolic Parameters

Body Composition

Naturally aged mice showed an age dependent decrease in their lean-mass to fat-mass ratio with the most significant difference between 3 months and 12 months (figure 2.1). When compared with vehicle treated mice, mice receiving DGal did not show any statistically significant changes in their body composition.

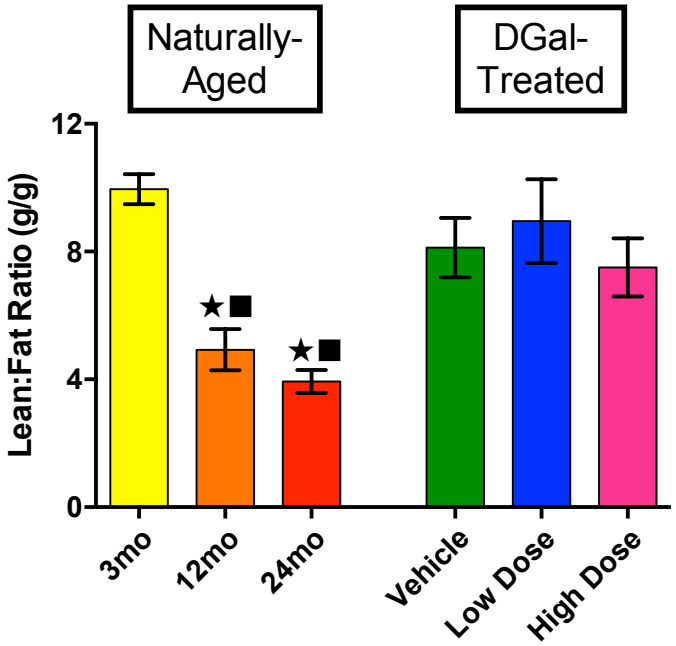

Figure 2.1. Lean vs. fat ratio for naturally-aged and 3mo DGal treated mice. MRI data describing absolute lean and fat mass processed to a percentage value of total body weight, ratios presented for percentages of total body weight. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. vehicle when analysed by one-way ANOVA.

Fast-Refeed Food Intake

No statistical significance was observed between any groups in the fast-refeed study when data was analysed by AUC (figure 2.2.B). However, a non-significant decrease in food intake was observed in ageing with no effect from DGal administration. Mice in the 24 months group were found to eat significantly less food than 3 months at 8 and 24h time points (figure 2.2.A). No differences were observed between 12 months and 3 months or between DGal and vehicle groups at any time.

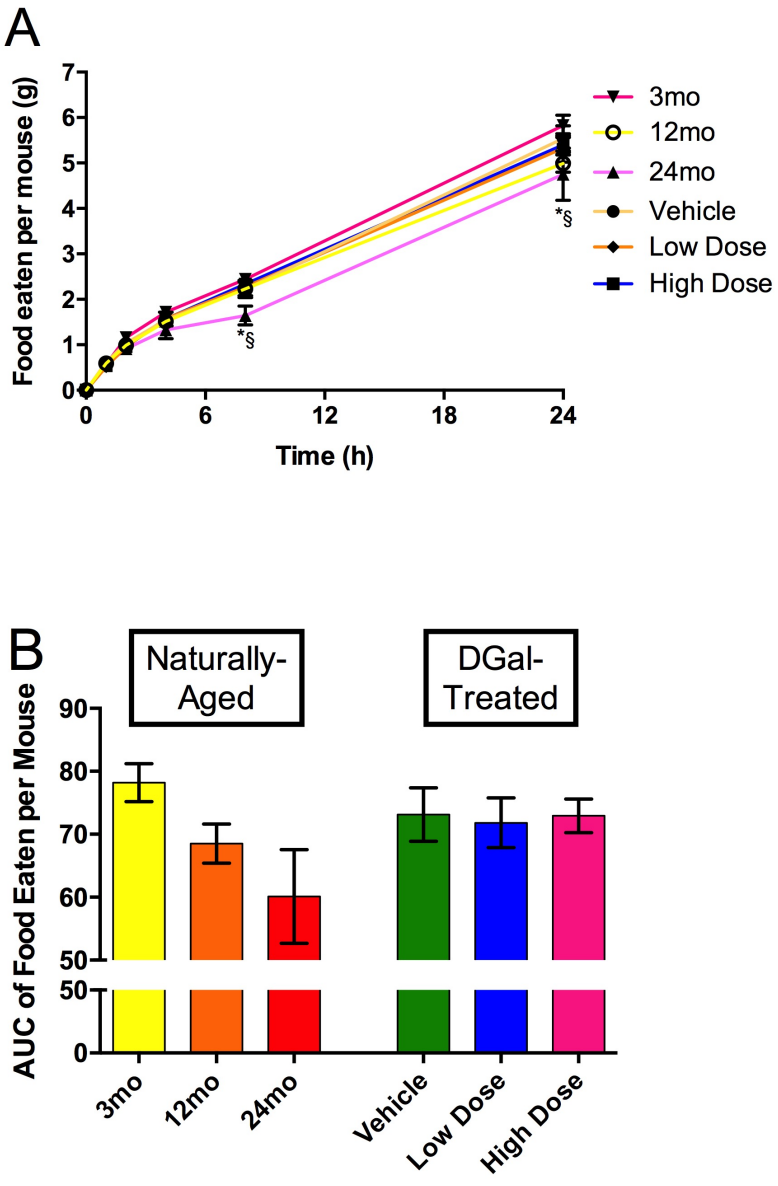

Figure 2.2. Fasting-refeed data over a 24h food intake study for naturally-aged and 3mo DGal treated mice. A=Mass of food against time progression for 24h. B=AUC analysis for A. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. § P≤0.05 vs. 3mo, * P≤0.05 vs. vehicle when analysed by two-way RM-ANOVA. Data presented as mean ± SEM.

Oral Glucose Tolerance-Test (oGTT)

Parameters measured for the oGTT were blood glucose and insulin. At 25min post glucose administration, 24 months mice had significantly lower increase blood glucose versus 3 months (figure 2.3.A). No differences were observed between 12 months and 3 months or between DGal and vehicle groups at any time (figure 2.3.A). Analysis showed a decrease in AUC (figure 2.3.B) with ageing but no difference with DGal administration.

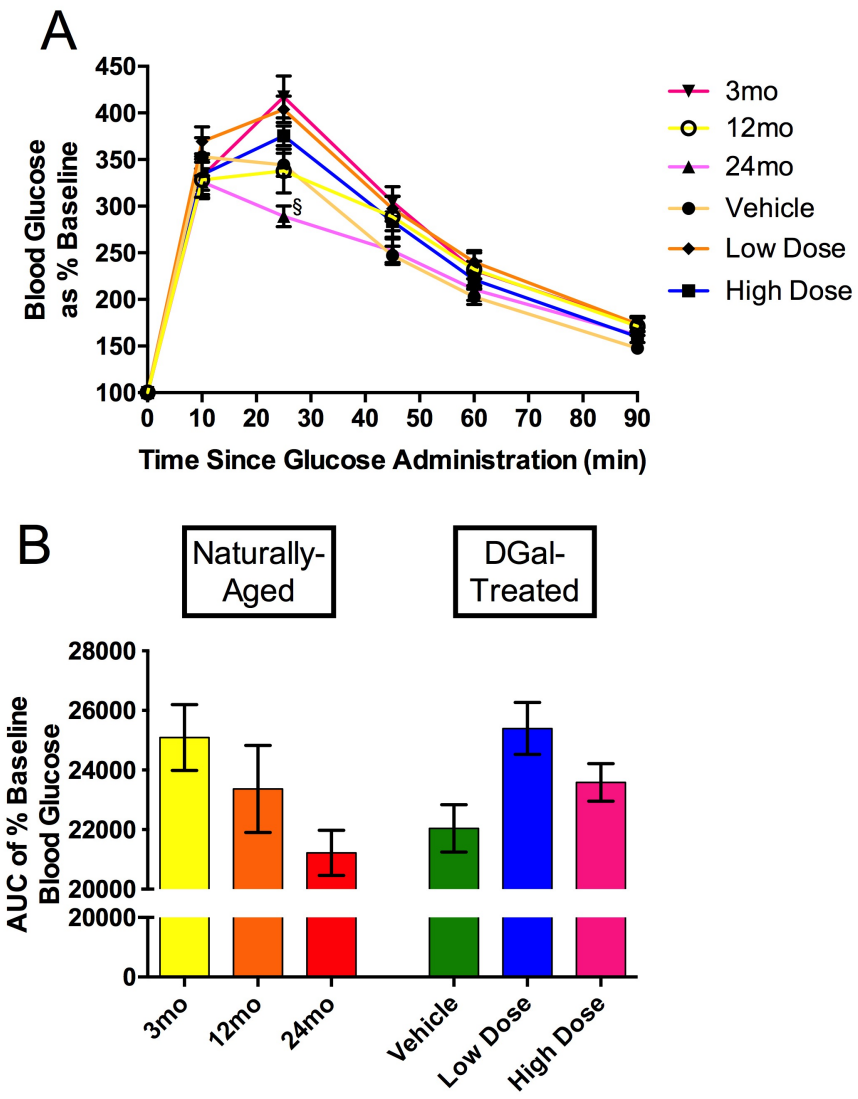

Figure 2.3. Time course progression of blood glucose for oGTT (A) and AUC analysis of data in 2.3.A (B) for naturally-aged and 3mo DGal treated mice. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. § P≤0.05 vs. 3mo when analysed by two-way RM-ANOVA. Data presented as mean ± SEM.

24 months and 12 months mice had a clear increase in blood insulin at 10 min post glucose administration that was not seen in any other group in figure 2.4.A. The data in figure 2.4.A could not be analysed by two-way RM-ANOVA due to missing data. Analysis of the data in figure 2.4.A showed that the AUC for 12 and 24 months mice was significantly increased versus 3 months with no change in DGal groups against vehicle (figure 2.4.B).

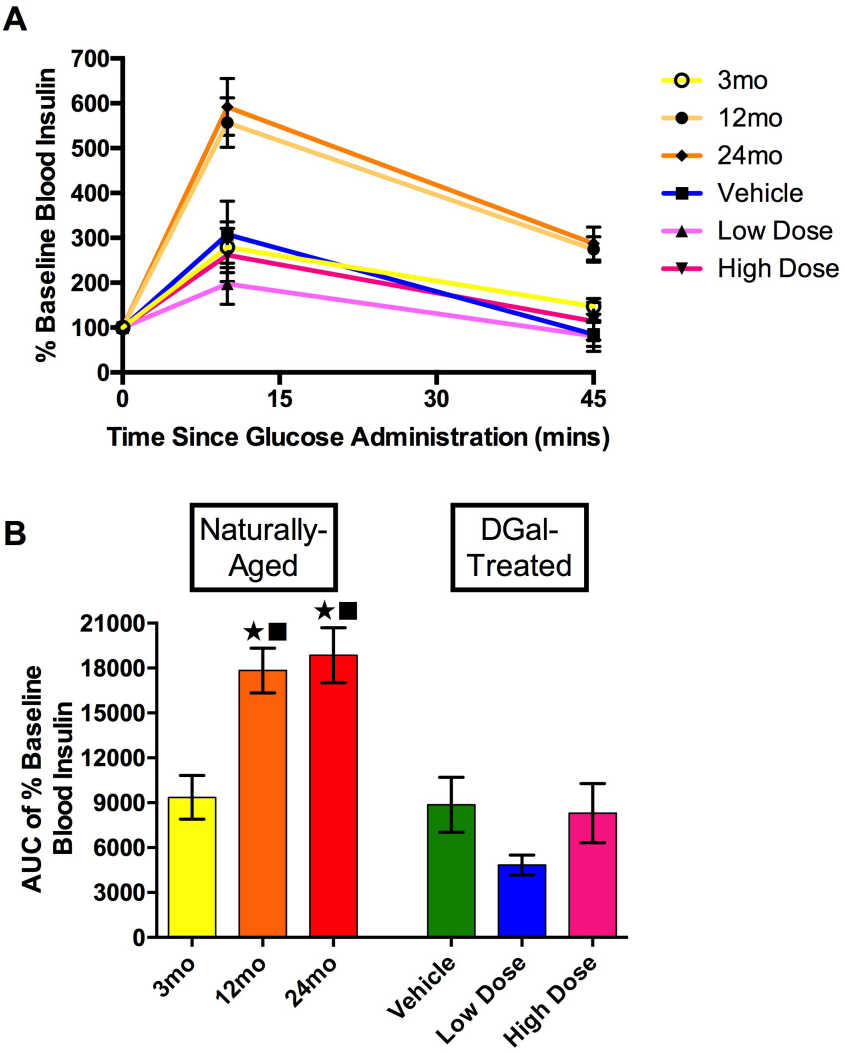

Figure 2.4. Time course progression of blood insulin for oGTT (A) and AUC analysis of data in 2.4.A (B) for naturally-aged and 3mo DGal treated mice. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. Data presented as mean ± SEM.★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Insulin Tolerance Test (ITT)

In this test, blood glucose for 24 months mice was significantly higher versus 3 months at 15, 45, 60 and 90 min and against vehicle at 45 and 60min. No difference was observed between 12 months and 3 months and DGal and vehicle throughout the test (figure 2.5.A). There was no change in AUC analysis between any groups; though there was a significant increase for 24 months versus vehicle groups, this appears to be irrelevant given variations in the data.

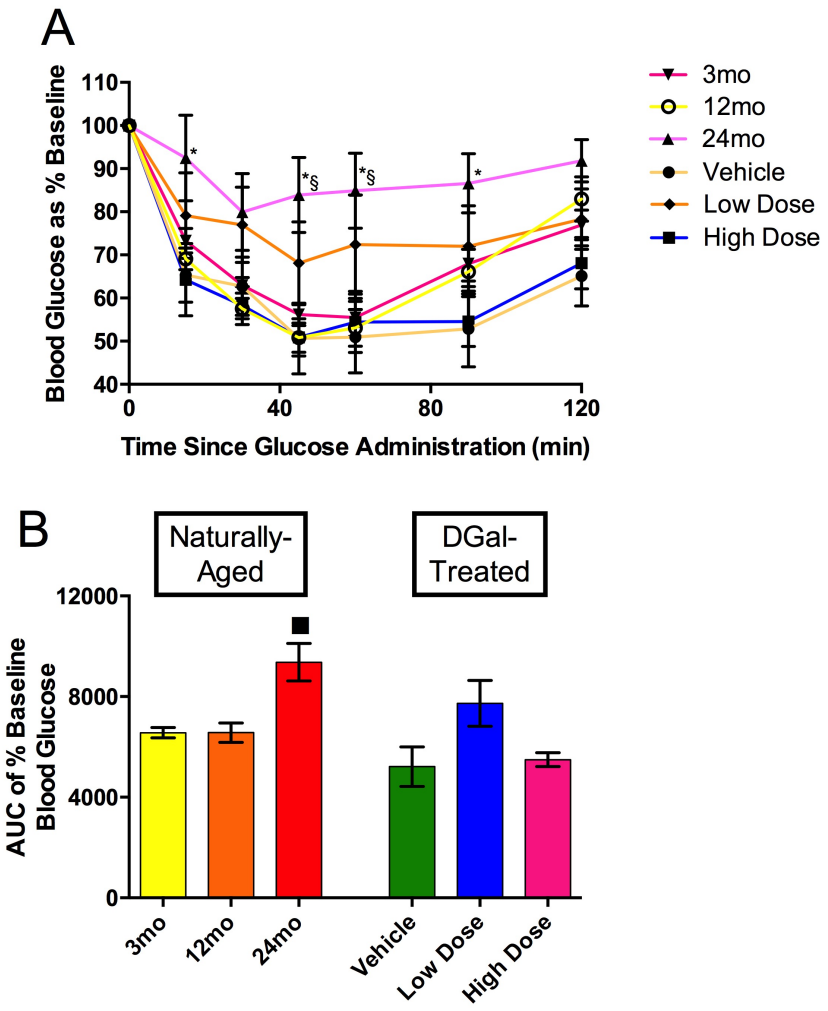

Figure 2.5. Time course progression of blood glucose for ITT (A) and AUC analysis of data in 2.5.A (B) for naturally-aged and 3mo DGal treated mice. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. § P≤0.05 vs. 3mo, * P≤0.05 vs. vehicle when analysed by two-way RM-ANOVA. ■P≤0.05 vs. Vehicle when analysed by one-way ANOVA. Data presented as mean ± SEM.

Lipid Profile

No changes in serum total cholesterol (figure 2.6.A) were observed between any groups. Serum HDL (figure 2.6.B) showed no statistically significant differences between any groups. Serum LDL (figure 2.6.C) was significantly elevated in 12 months and 24 months groups versus 3 months. LDL was elevated in high dose DGal versus 3 months but not vehicle. HDL:LDL ratio (figure 2.6.D) shows an age-dependent increase towards the LDL becoming significant at 24 months with no changes in DGal groups versus vehicle.

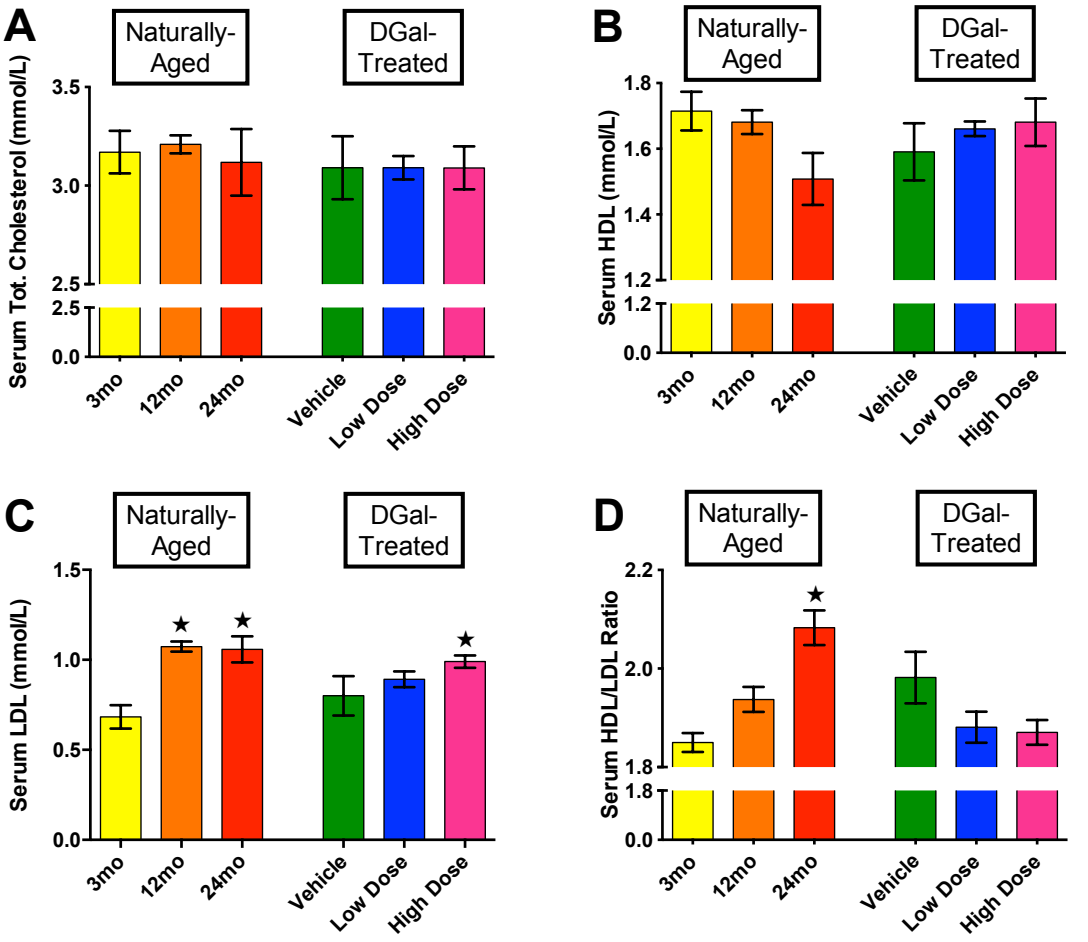

Figure 2.6. Serum lipid profiles for aged and 3mo DGal treated mice for naturally-aged and 3mo DGal treated mice. Total Serum Cholesterol (A), Serum High-Density Lipoprotein (HDL) (B), Serum Low-Density Lipoprotein (LDL) and HDL/LDL ratio. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo when analysed by one-way ANOVA.

Full Blood Count (FBC) and White Blood Cell (WBC) Differential

In figure 2.7.A we see that there is an increase in total white blood cell (WBC) count with age becoming significant against 3 months at 24 months. The same increase is seen in all differentials (figures 2.7.B, C & D) with granulocytes (figure 2.7.D) also significantly increased at 12 months versus 3 months with a further increase to 24 months. Total WBC count (figure 2.7.A) did not show any change with DGal administration, nor did any of the differentials (figures 2.7.B, C & D).

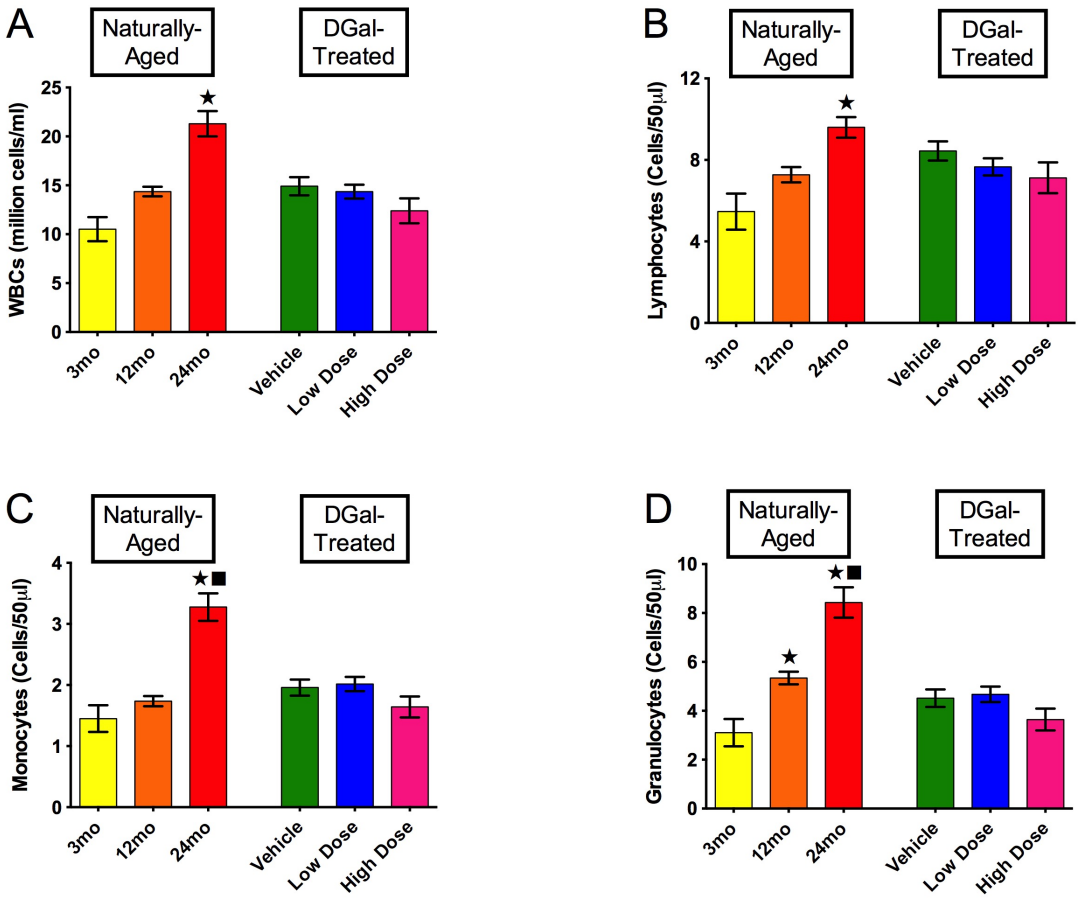

Figure 2.7. Full Blood Count with white cell differential for naturally-aged and 3mo DGal treated mice. A=WBC Count. B=Lymphocyte Count. C=Monocyte Count. D=Granulocyte Count. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Serum Cytokines

Keratinocyte chemoattractant (KC) displayed an elevation at 24 months that was non-significant against 3 months but was significant against vehicle. No change in KC level was observed with administration of DGal versus vehicle or between 3 and 12 months.

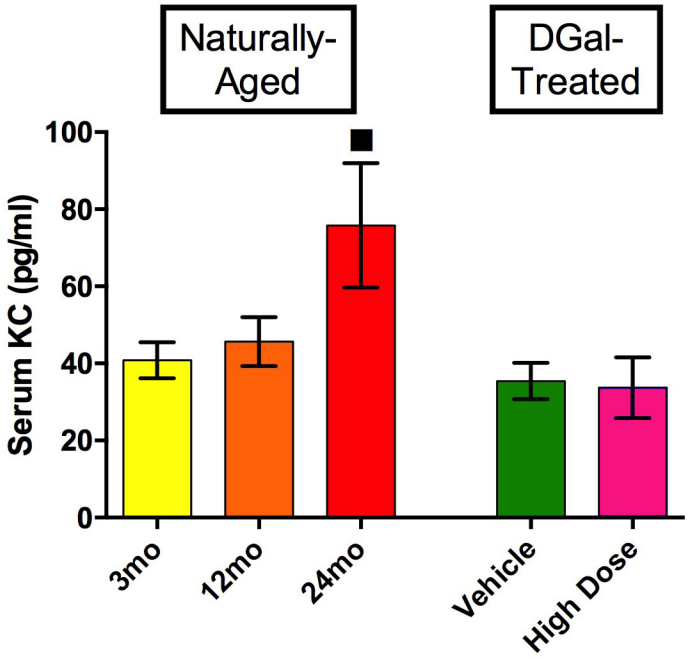

Figure 2.8. Serum mouse KC analysis for naturally-aged and 3mo DGal treated mice. 3mo: n=8, 12mo: n=8, 24mo: n=7, vehicle: n=8, high dose: n=8. Data presented as mean ± SEM. ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Tissue Cytokines

Renal Tissue Cytokine Protein Levels

No changes were observed between any groups in levels of either IFNγ (figure 2.9.B) or TNFα (figure 2.9.D). IL-1β (figure 2.9.A) showed an increase with age being significant against 3 months at 12 months and increasing further at 24 months. No change in DGal treated animals IL-1β was observed. KC (figure 2.9.C) was found to be elevated with age becoming significant against 3 months at 24 months and no change in KC for DGal animals.

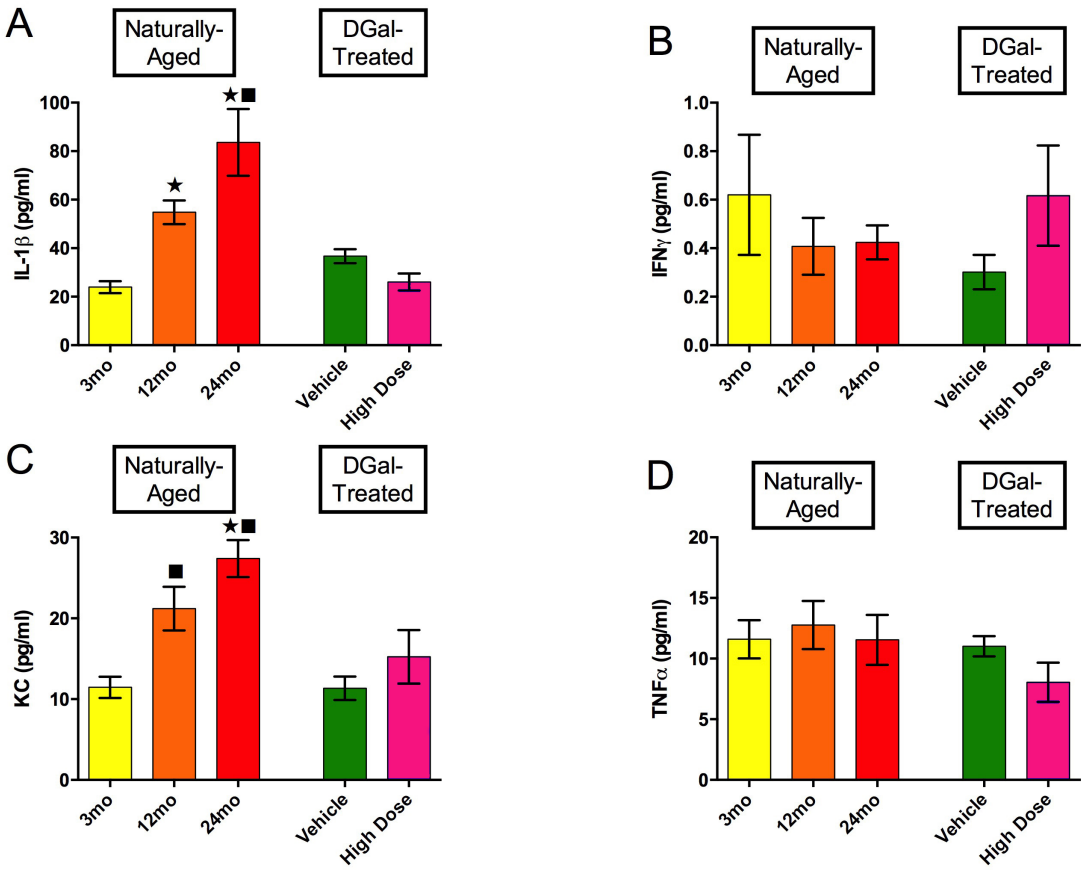

Figure 2.9. Protein cytokine analysis for lysed kidney tissue samples for naturally-aged and 3mo DGal treated mice. Cytokines analysed were IL-1β (A), IFNγ (B), KC (C) and TNFα (D). 3mo: n=8, 12mo: n=8, 24mo: n=8, vehicle: n=8, high dose: n=8. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Renal Tissue Cytokine Gene Expression

Following the protein data found in the kidney, gene expression levels for cytokines in the kidney were established. Expression of IL-1β (figure 2.10.A), transforming growth factor-β (TGFβ) (figure 2.10.B) and TNFα (figure 2.10.C) all increased with age, becoming significant against 3 months at 24 months. DGal had no effect on the expression of IL-1β, TGFβ or TNFα in renal tissues regardless of dose.

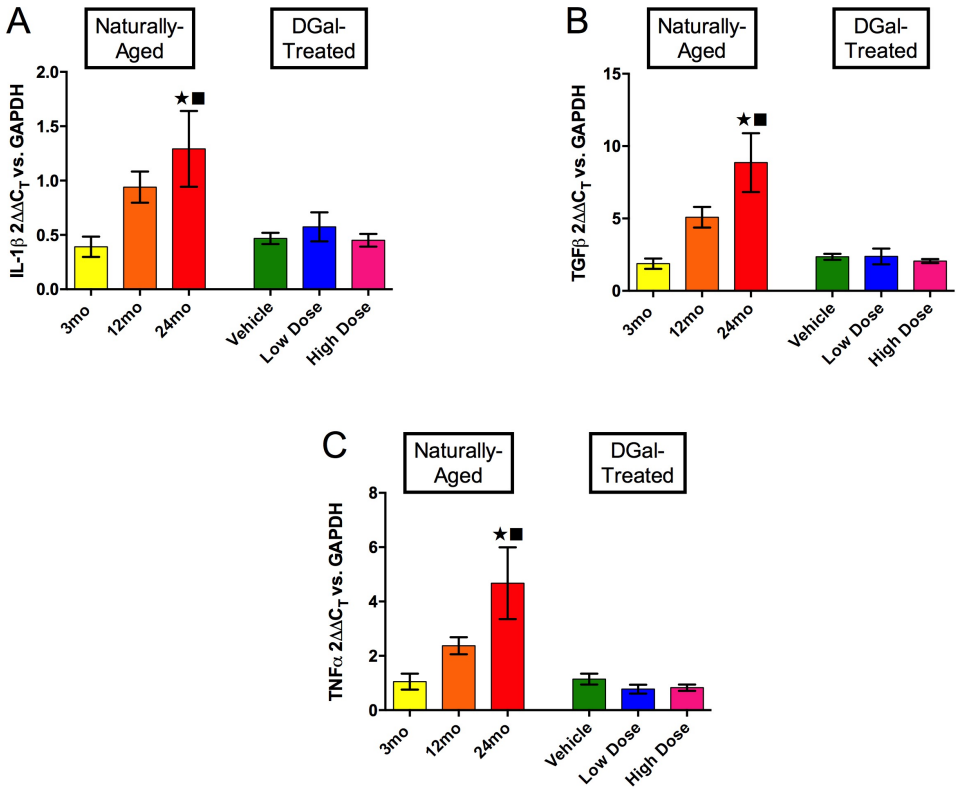

Figure 2.10. $2\Delta\Delta C_T$ values for expression of IL-1β (A), TGFβ (B) and TNFα (C) for naturally-aged and 3mo DGal treated mice. 3mo: n=12, 12mo: n=12, 24mo: n=12, vehicle: n=12, low dose: n=12, high dose: n=12. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Hepatic Tissue Cytokines

The two cytokines detected in the liver were TNFα (figure 2.11.A) and IL-1β (figure 2.11.B), these two showed no significant changes between any groups. However IL-1β (figure 2.11.B) showed an increasing trend with age that was non-significant.

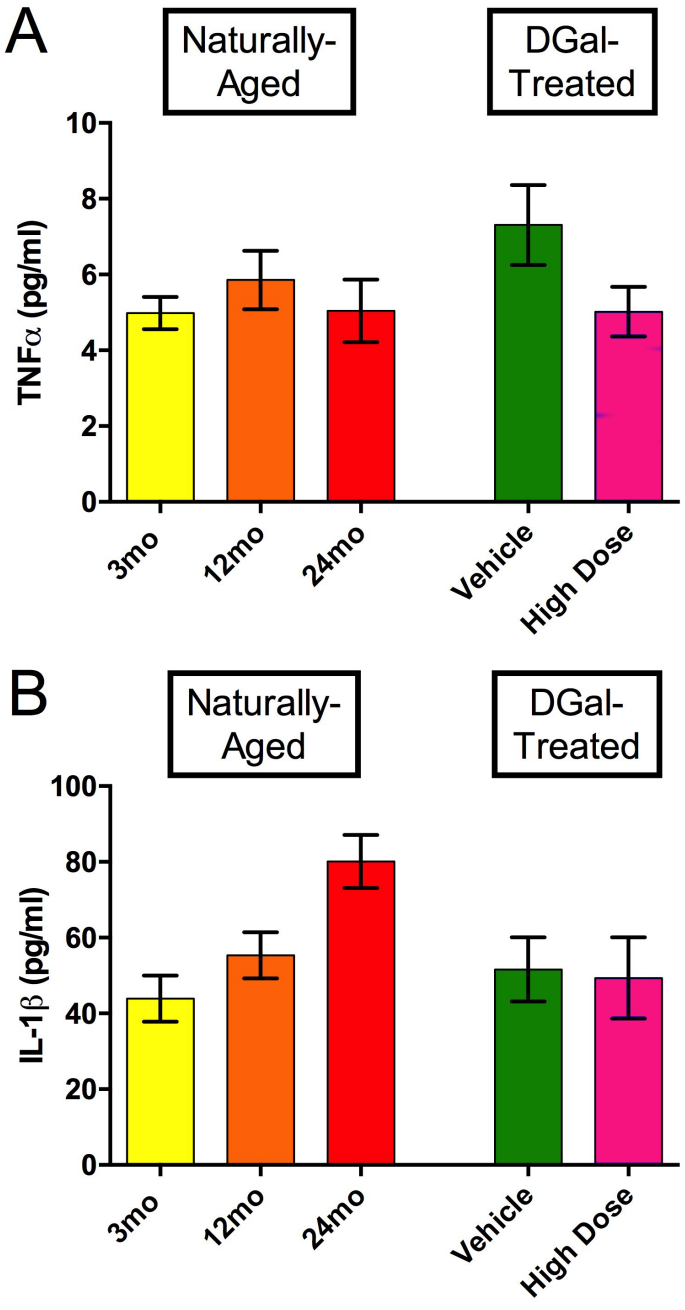

Figure 2.11. Protein cytokine analysis for lysed Liver tissue samples for naturally-aged and 3mo DGal treated mice. Cytokines analysed were TNFα (A) and IL-1β (B). 3mo: n=8, 12mo: n=8, 24mo: n=8, vehicle: n=8, high dose: n=8. Data presented as mean ± SEM.

Lung Tissue Cytokines

The only cytokines found to be at a detectable level in the lung were IL-1β and KC, these two cytokines showed a similar response to ageing. Both IL-1β (Figure 2.12.A) and KC (figure 2.12.B) have increasing levels in the lung with age, becoming significantly elevated against 3 months at 12 months and further significantly increased at 24 months. DGal administration had no effect on either of these levels.

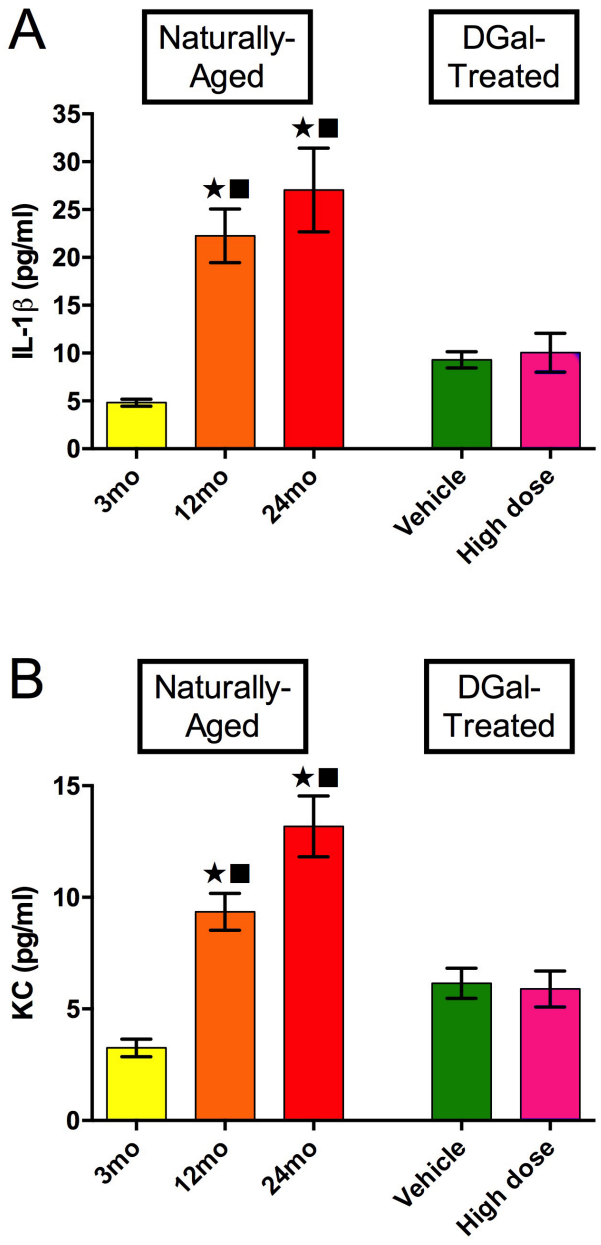

Figure 2.12. Protein cytokine analysis for lysed lung tissue samples for naturally-aged and 3mo DGal treated mice. Cytokines analysed were IL-1β (A) and KC (B). 3mo: n=8, 12mo: n=8, 24mo: n=8, vehicle: n=8, high dose: n=8. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Skeletal Muscle Cytokines

The only cytokine found to be detectable in the skeletal (Quadriceps) muscle was TNFα. TNFα shows an increasing trend with age, becoming significant at 24 months against 3 months. There was no significant difference observed between DGal treated and vehicle treated animals in this test.

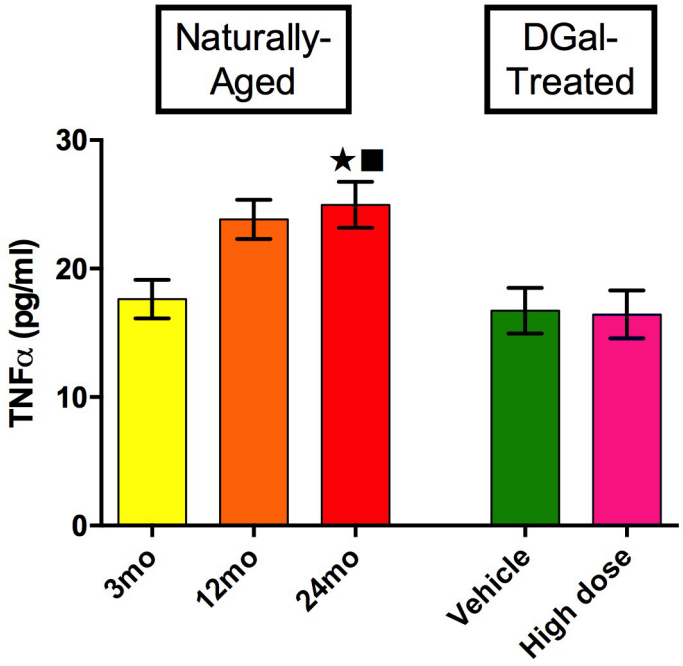

Figure 2.13. Protein TNFα analysis for lysed quadriceps tissue samples for naturally-aged and 3mo DGal treated mice. 3mo n=8: 12mo n=8: 24mo: n=8, vehicle: n=8, high dose: n=8. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Renal Impairment and Pathology

Urine ELISAs

No significant change between any groups was found in any of the urine tests carried out.

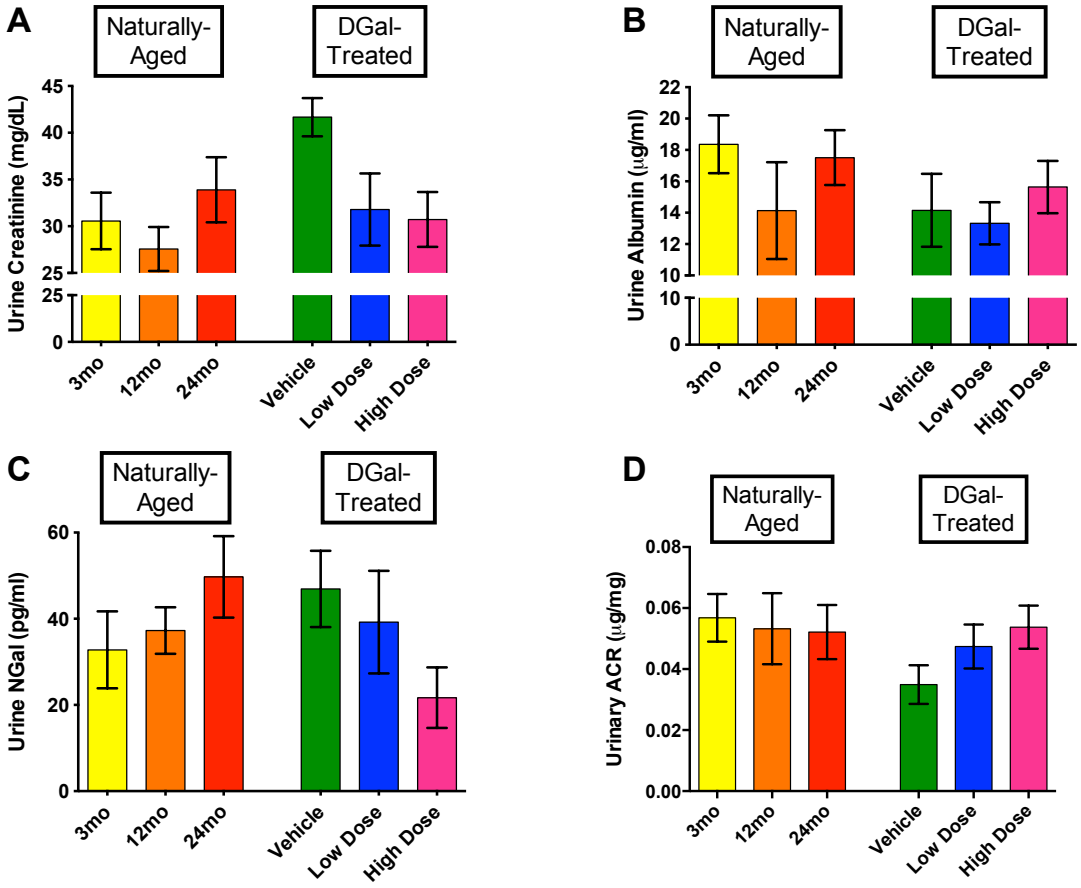

Figure 2.14. Urine ELISA analysis for Creatinine (A), Albumin (B), Neutrophil Gelatinase (N-Gal) (C) and Albumin/Creatinine Ratio (ACR) (D) for naturally-aged and 3mo DGal treated mice. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. Data presented as mean ± SEM.

Serum Markers of Renal Function

Serum urea (figure 2.15.A) showed no change with age but declined with increasing doses of DGal, with this decrease becoming significant against vehicle and 3 months with high dose administration. Serum creatinine (figure 2.15.B) was unaffected by ageing but showed a dose-dependent decrease in animals treated with DGal which was significant against vehicle and 3 months in animals treated with high dose. When creatinine is normalised for muscle mass of individual animals (figure 2.15.C) there is a significant increase in serum creatinine in both 12 months and 24 months mice versus 3 months. DGal administration showed no significant increase in normalised creatinine versus vehicle control.

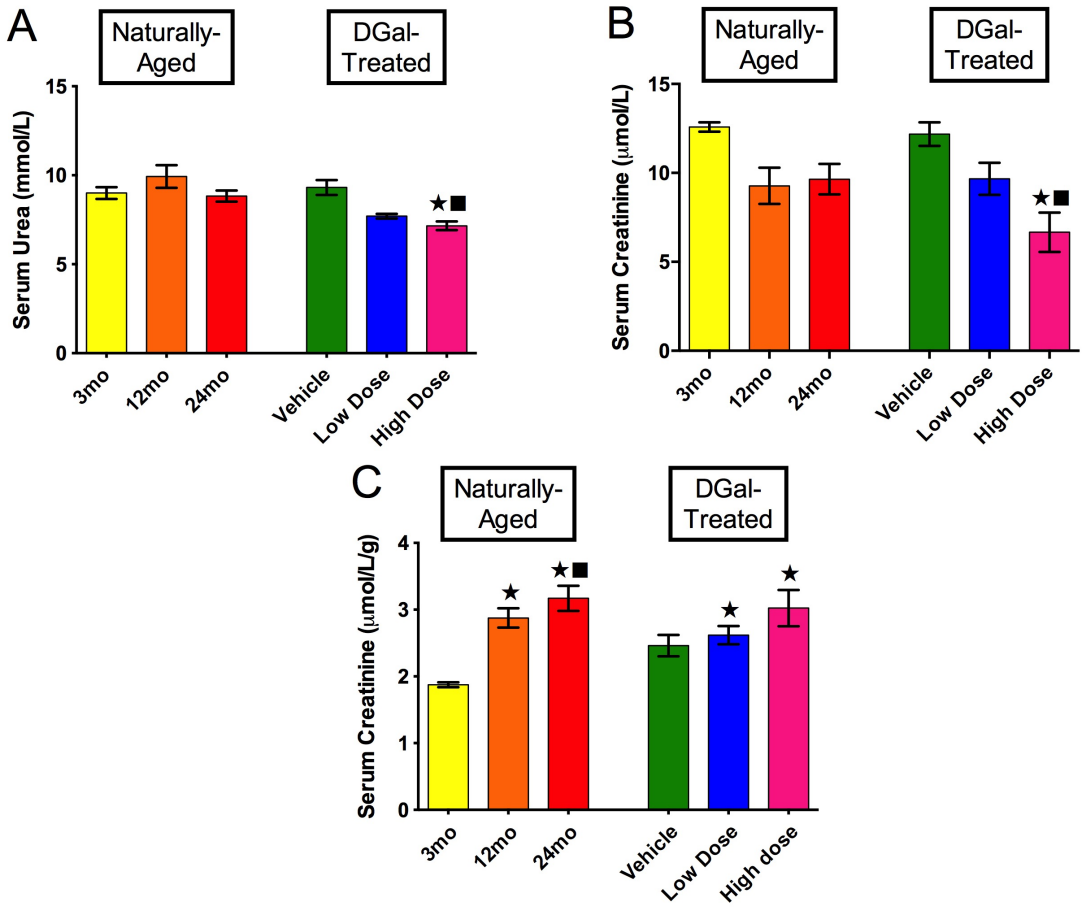

Figure 2.15. Serum measurements of creatinine (A) and urea (B) as well as serum creatinine normalised for lean mass (C) for naturally-aged and 3mo DGal treated mice. 3mo: n=12, 12mo: n=12, 24mo: n=14, vehicle: n=12, low dose: n=12, high dose: n=12. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, ■ P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Renal Tissue Markers of Protein Homeostasis

Tissue ELISA Analysis for Effects of Protein Homeostasis.

Protein carbonylation (figure 2.16.A) in 12 months animals was found to be significantly increased versus 3 months but no significance was found between any other groups. Advanced glycation end-products (AGE) (figure 2.16.B) showed no change in their levels in renal tissue between any groups.

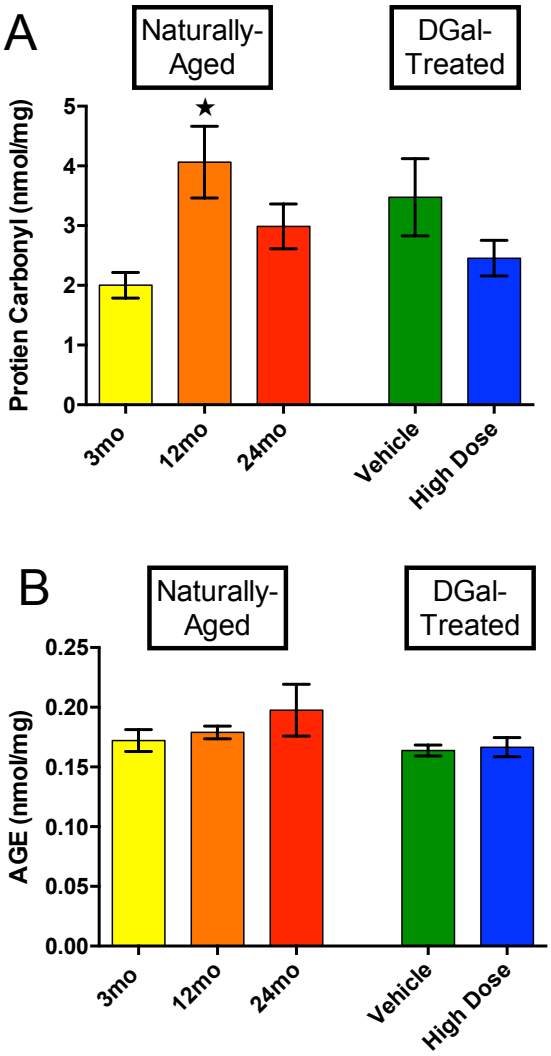

Figure 2.16. Protein carbonyl (A) and AGE (B) content for kidney tissue in both naturally aged and 3mo DGal treated mice. 3mo: n=8, 12mo: n=8, 24mo: n=8, vehicle: n=8, high dose: n=8. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo when analysed by one-way ANOVA.

Tissue PCR Analysis for Markers of Oxidative Stress

NADPH oxidase enzymes (Nox) were measured by qRT-PCR. Nox-1 (figure 2.17.A) shows no significance between any groups though both ageing and increasing doses of DGal do appear to increase the expression of this enzyme, with the effect more profound in DGal treated animals. Nox-2 (Cybb) (figure 2.17.B) is increased in an age-dependent manner becoming significant against 3 months at 24 months with no changes observed in DGal treated mice.

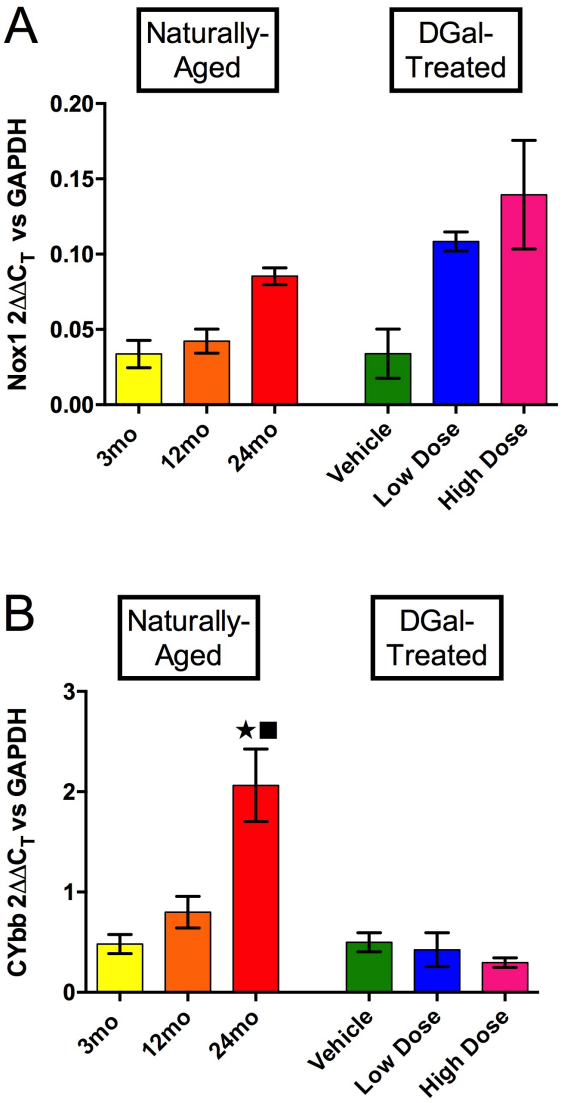

Figure 2.17. Expression data for Nox enzymes, Nox-1 (A) and Cybb (Nox-2) (B) for naturally-aged and 3mo DGal treated mice. 3mo: n=4, 12mo: n=4, 24mo: n=4, vehicle: n=4, low dose: n=4, high dose: n=4. Data presented as mean ± SEM. ★ P≤0.05 vs. 3mo, n P≤0.05 vs. Vehicle when analysed by one-way ANOVA.

Renal Histology Analysis

Our studies have shown that there is a marked and significant change in the size of both the glomerulus and the Bowman's capsule with ageing. No data is available for DGal treated mice. Representative images of the renal corpuscles analysed are shown in figure 2.18. Both glomerular size and Bowman's capsule expansion increase from 3 months (figure 2.18.A) to 12 months (figure 2.18.B) and further increasing at 24 months (figure 2.18.C). There is very little Bowman's capsule visible in figure 2.18.A with more visible in 2.18.B and even more in 2.18.C. The increase in glomerular size is less easily viewed in figure 2.18 but is quantified in 2.19.A.

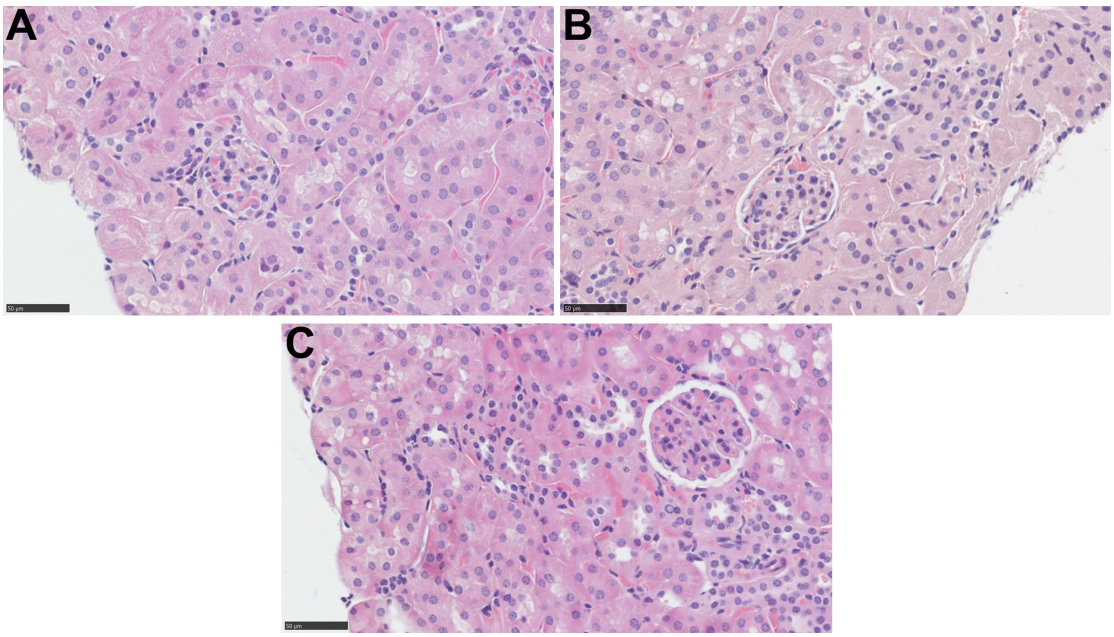

Figure 2.18. Representative images of glomeruli assessed to create the data in figure 2.20. A= 3mo, B= 12mo and C= 24mo. Scale bar is 50µm.

In figure 2.19.A there is an increase in glomerular area with age, becoming significant against 3 months at 12 months and continuing to be further significant against both 12 and 3 months at 24 months. In figure 2.19.B there is an increase in Bowman's capsule area with age, becoming significant against 3 months at 12 months and continuing to be further significant against both 12 and 3 months at 24 months. Figure 2.19.C presents the area of the Bowman's capsule as a percentage of the total area of the renal corpuscle, this shows that there is an age-dependent increase in the area of the Bowman's capsule becoming significant against 3 months at 24 months, this showed significance between 12 and 24 months.

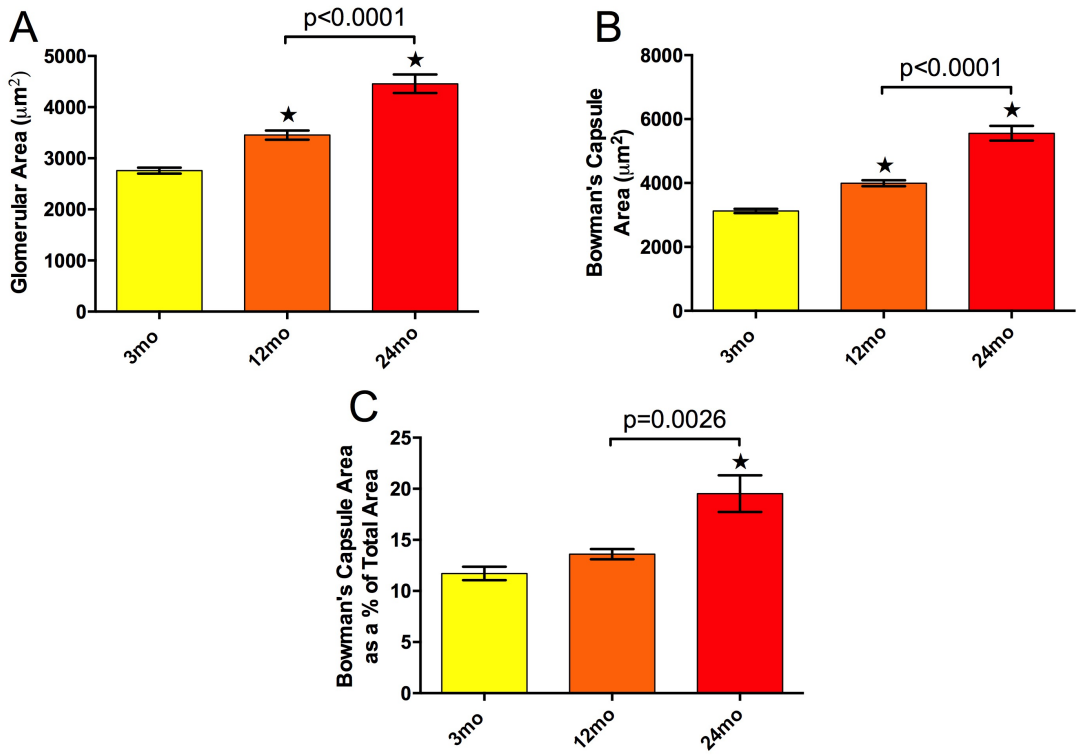

Figure 2.19. Histological analysis of glomeruli from aged mice. A, glomerular area. B, Bowman's capsule area. C, % of total area occupied by Bowman's capsule B. 'Biological n'- 3mo: n=12, 12mo: n=12, 24mo: n=12. 'Datapoint n' - 3mo: n=240, 12mo: n=240, 24mo: n=240. Data presented as mean of all mice for the means for 20 randomly selected glomeruli per mouse ± SEM. ★ P≤0.05 vs. 3mo when analysed by one-way ANOVA.

Discussion

To assess the effectiveness of the DGal model, experiments were planned around the work of Lopez-Otin et al., (2013). These experiments are designed to make a comparison of natural ageing versus administration of DGal. We used increasing age and low/high dose of DGal to create two 'dose-response curves' that can be compared to one and other. The hope was to be able to use the 'age dose-response curve' and 'fit' the DGal dose to that for various parameters, showing their 'age'. In order to break down the whole picture of these investigations, I will group similar experiments together to assess differing aspects before uniting these to make final conclusions.

Nutrient Sensing and Metabolic Profile

This area was assessed initially by body composition, this is a key marker given the role of fat storage in insulin sensitivity and metabolic activities (Ravussin & Smith, 2002). The increased storage of fat combined with failure of insulin sensing shown both by OGTT and ITT tests means that these experiments suggest animals show some form of metabolic syndrome with advanced age. The insipid insulin resistance seen with age is similar to that seen in the clinical condition of prediabetes (Serrano-Rios et al., 1970). DGal treatment has not yet been suggested to have a link to diabetes; these findings that DGal does not have a similar effect to ageing with regard to metabolic profile are not surprising.

When we look on to the lipid profile of these animals it is evident that there is hypercholesterolaemia in the aged animals. Considering the link between diabetes, prediabetes and hyperlipidaemia it is perhaps not surprising that these have come together in these aged animals. It appears that these animals have developed a 'metabolic syndrome' when these symptoms are united into one conclusion (Smith & Singleton, 2006). The mice treated with DGal did not experience the same level of hypercholesterolaemia, however this

was only in HDL. HDL/LDL levels appear to be reduced overall towards the HDL with DGal treatment. It is known that a shift toward LDL cholesterol, like in ageing is associated with a higher risk of CVD with the inverse being cardioprotective (Lemieux et al., 2001).

Ultimately, the combined insulin resistance and hypercholesterolaemia observed in aged animals as an increasing trend is a metabolic syndrome. This metabolic syndrome observed in aged mice was not observed in DGal treated mice that remained almost unchanged versus controls. DGal administration does not have a link to diabetes based on these results.

Cellular Communication and Inflammation and Oxidative Stress

These investigations have found an increase in the markers of inflammation with ageing. The increases in inflammatory markers like raised WBC count and pro-inflammatory cytokines were modest and as a rule showed an increase with age in the characteristic 'age-response curve' which has been evident in many sets of results presented here. These results in themselves are not surprising and follow the well-established principle that ageing is associated with low-level chronic inflammation (Franceschi & Campisi, 2014).

What is interesting regarding these results is the source and cause of the pro-inflammatory markers we see in these animals, though the source is unclear from our investigations. Cellular death and damage which is linked to release of inflammation-causing Damage Associated Molecular Patterns (DAMPs) like HMGB1 has been suggested as a promoter of this inflammation (Franceschi et al., 2000). Induction of inflammation by this mechanism is well established (Zhang et al., 2010) and it is suggested that a failure to resolve this inflammation could be a cause of some sort of cumulative inflammation (Oishi & Manabe, 2016).

Another possible cause of this inflammation is mitochondrial dysfunction, which can be linked to the induction of cellular death in its own way (Iyer et al., 2013). The investigations presented here show that there is a change in the function of the mitochondria of aged animals but that that appears to no longer be the case in very advanced age at 24 months. A highly probable cause and one which links to the administration of DGal is that of oxidative stress induced inflammation. It is known that ROS can induce cytokine expression (Mittal et al., 2014) and when the results here presented pertaining to the expression of Nox enzymes (Lan, Kisseleva & Brenner, 2015) in the renal tissues of aged animals are considered there is an increase in Nox-1 and 2 expression which follows the trend shown in many cytokines. Interestingly, there is a dose-response increase in Nox-1 with DGal treatment, Nox-1 is known to play a role in the induction of inflammation (Singel & Segal, 2016, Zhang et al., 2009) but there is no increase in inflammatory markers seen in the DGal treated groups. It is possible that this is evidence that oxidative stress, here shown as Nox enzyme expression, is secondary to inflammation. Inflammation is evident in aged animals with oxidative stress but not in the DGal treated animals, which also have oxidative stress.

Further oxidative stress makers, in the form of protein carbonylation show that there may be an increase in protein oxidation with age, though the level has returned to normal at 24 months, this could be due to the insolubility of these types of proteins and therefore better methods to analyse this may be needed. No change in this is seen in DGal treated animals, so it could also be suggested that inflammation may be as a result of increased oxidative stress but the type or amount of oxidative stress induced by DGal is not wide ranging enough to result in the sorts of inflammation seen in ageing.

Glycation is not strictly oxidative stress but is seen in disorders and models with high levels of oxidative stress like the DGal model (Song et al., 1999) and diabetes (Basta, Schmidt & De

Caterina, 2004). AGE has been seen in the DGal model in the brain both in the literature (Song et al., 1999) and in experiments carried out by colleagues in the CNS division at Takeda Cambridge Ltd. shown in figure 2.20. These results show, interestingly that there appears to be no accumulation of AGE in the kidneys with DGal treatment and only an extremely small upwards trend in these with age. It is known that AGE increases in the brain with natural ageing (Ramasamy et al., 2005) so for this to be missing in ageing in the ageing kidney and in DGal treated kidneys suggests that the kidney as an organ is not so susceptible to proteins becoming glycated.

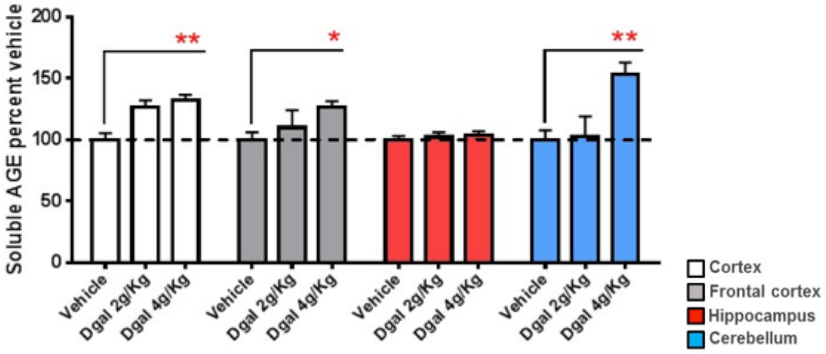

Figure 2.20. AGE in differing areas of the brain with DGal treatment (from Chadwick et al., under review).

The other aspect of inflammation here is the question of the source of inflammation. Although these investigations were focused on the kidney it was important to explore the effect of holistic inflammation on the whole body. Initial indications in the kidney showed the presence of a number of cytokines in the aged kidney including, importantly, KC. Keratinocyte chemoattractant was found to be elevated in the serum of aged animals as well as the kidney but when qRT-PCR was carried out on RNA extract from aged animals it was not found at a detectable level. This suggests that KC is exogenous to the kidney in ageing in spite of it being linked strongly to senescent cells (Freund et al., 2010). Further investigations showed that KC was also present in the lung, but not in liver or skeletal muscle. The reason for the differing distribution of KC is unclear, it could be suggested that it is due to high

blood flow as both kidneys and lungs appear to have high protein levels, as does the circulation. The lack of KC presence in the liver which also has a very high blood flow would suggest that circulatory effects are not a major driver of this tissue distribution. Ultimately, without protein and gene expression data from all tissues of the body it would be hard to establish the source of KC within the ageing mouse.

Renal Function

The investigations in this chapter were ultimately aimed at assessing the ability of DGal to induce renal ageing for research modelling purposes. The major factor which is important to model when looking at renal ageing is any change in renal function. Changes in renal function are well known to be linked with ageing (O'Hare et al., 2007, Davies & Shock 1949) and so, establishing what form these take in mice and if they are replicated with DGal administration was important. DGal is known to cause renal injury in its own right (Fan et al., 2009) so it was also important to assess the effects of natural ageing on the kidney in order to make comparisons.

Key clinical markers of renal damage are serum creatinine and urea (Schwartz et al., 1976, Morgan, Carver & Payne, 1977), elevated creatinine is a marker of renal failure and urea is commonly elevated in renal injury indicating poor healthcare outcomes (Parfrey et al., 1996). Serum creatinine in aged and DGal animals was found to be elevated when normalised for muscle wastage, which was important in this study, given the very different lean masses of young and old animals (Cockcroft & Gault, 1976). There is some sort of renal impairment in these animals; this may be associated with a hyperfiltration seen in early stage renal failure (Palatini, 2012) and can be a risk factor for the development of more serious renal disease (Helal et al., 2012). There is no significant change in serum levels of urea with age, however a lowering of serum urea is seen in DGal mice. The lower amount of

urea in the serum of DGal treated mice could be suggestive of tubular toxicity of DGal; urea is approximately 50% reabsorbed in the proximal tubule (Torrtora & Derrickson, 2011). Lowered serum levels of urea are suggestive of the process of reabsorption being carried out less efficiently (Goldstein, Lenz & Levitt, 1969). Ageing can be considered a glomerulopathy (dysfunction of the glomerulus) (Rosenberg et al., 2002) and the lack of change in urea could be indicative that there is little or no tubular pathology. DGal however has shown some evidence of tubular pathology meaning that there is an additional renal damage that is not seen in natural ageing making this model incompatible with modelling ageing.

Urinary markers of renal injury in this study showed no significance in any parameter, however some trends were evident. The key urinary marker of glomerular function, ACR was unchanged with age, likely to mean that if there is renal injury that it is sub-clinical. ACR in DGal treated mice was increased versus vehicle control, though this may have been as a result of a very high creatinine within this group and none of these findings were statistically significant. N-Gal is a marker of renal inflammation and damage (Mishra J et al., 2005); it appears to be increasing with age, though not significantly due to a high data spread. Urinary markers of renal injury may not be the best way to measure this type of renal injury, as it could be sub-clinical but still describe a pre-disposal to more serious renal disease.

By looking at the ageing kidney we can see some pathological changes to the structure of the glomerulus. It appears from our investigations that these changes have not yet begun to show any sort of clinical syndrome but this could indicate a predisposition to harsher injury in future, following with the conclusions of O'Hare et al., (2007).

Final Conclusions

Ultimately, these investigations have concluded that the DGal model is not suitable for modelling of whole-body ageing. The lack of inflammation, which is either a marker or driver of ageing, in the kidney or indeed any other organs with DGal treatment means that it is hard to believe DGal has any effect on the body outside of the CNS. For this model to be used for renal ageing studies specifically it is important that if there is any renal injury induced, that it be mimetic to that induced by ageing. The renal injury seen with DGal treatment seems to not be similar enough to that of ageing and is in fact a pathology in its own right. Animal models of disease are never perfect as it is almost impossible to recreate an exact pathology artificially in an animal so it is important that we use models appropriately and understand their shortcomings. It seems that the DGal model does induce an Alzheimer's-like pathology (Song et al., 1999) as has been observed in the work by Chadwick et al., (Under review) seen in figure 2.21. The most appropriate application of this model seems to be as a CNS specific model of neurodegeneration and that it is in fact not, as it has been called before, a model of advanced age.

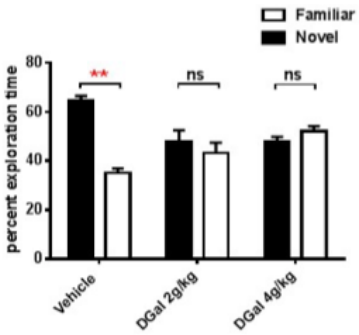

Figure 2.21. Data for novel object recognition, a marker of cognition, for animals treated with DGal (from Chadwick et al., under review).

Chapter III: Baricitinib in Diabetic Nephropathy

Introduction

The oral Jak1/2 inhibitor baricitinib, developed for RA was brought to market by Eli Lilly. The drug has since been approved by the food and drug administration (FDA) and European medicine agency (EMA) for use in clinic for RA; however, it has been suggested that it could have beneficial effects in DN. This suggestion was as a result of earlier studies showing a marked activation of the Jak/STAT pathway in patients with DN (Brosius, Tuttle & Kretzler, 2016). Indeed, our own results (Chapter IV) show that there is a sharp increase in expression of the Jak/STAT machinery and evidence of activation in a number of pro-inflammatory states within the kidney.

In RA Jak/STAT inhibitors lower activation of the cell (Goldstein et al., 2016) and humeral (Wang et al., 2014) mediated immune systems by blocking signal transduction of cytokines. Importantly certain cytokines are key in its activation IL-6 (Heinrich et al., 2003), IL-2 (Goldstein et al., 2016) and IL-4 (Wang et al., 2014) among others. This leads to the initial thought that this drug may act to prevent their downstream action and even upstream cytokine production, in short, a strong generalised anti-inflammatory (Brosius, Tuttle & Kretzler, 2016).

One of the key cytokines affected by Jak/STAT inhibition in RA is IL-6, which has also commonly been implicated in the pathogenesis of DN (Dalla Vestra et al., 2005). Specifically, it has been noted that IL-6 interacts with the cells of the glomerulus, the podocyte (Nosadini et al., 2000). Podocytes are known to be key to the integrity of the basement membrane and have been implicated heavily, along with mesangial cells (Mauer et al., 1984) in the pathogenesis of DN (Susztak et al., 2006). Mesangial expansion results in the ultimate occlusion of the afferent blood vessels and in ischaemia of the glomerulus (Abrass, 1995). Both podocyte and mesangial damage have been postulated, due to the changes they

undergo during DN, to be abrogated with use of Jak inhibitors (Marrero et al., 2006, Ortiz-Muñoz et al., 2010). It is postulated that the potential efficacy of this class of drugs is due to its ability to suppress the immune activation that plays a role in the pathology of DN (O'Shea et al., 2013).

It may be that the potential efficacy of drugs such as baricitinib could in fact be rooted in an alternative pathway other than simply the transduction of cytokine signals to activate immune cells. Jak/STAT signaling is heavily involved with cell survival and proliferative pathways as it signals for a number of growth factors and cytokines, like TGFβ and TNFα which can modulate cell cycle progression (Lovibond et al., 2003). It has specifically been suggested that the Jak/STAT pathway is involved in the pathogenesis of diabetic glomerulopathy and mesangial expansion (Berthier et al., 2009).

Downstream of Jak and STAT activation is the upregulation of the suppressors of cytokine signalling (SOCS). SOCS role is, as the name might suggest, to act as a negative feedback on cytokine signalling initiated by Jak/STAT activation (Yoshimura, Naka & Kubo, 2007). The mechanism of this is shown in figure 3.1 where by binding of a cytokine to its receptor recruits Jak that in turn phosphorylates STAT causing transcriptional changes, in this case expression of SOCS. SOCS proteins aid in the ubiquination and degradation of both Jak and STAT proteins, reducing the signal transduction capacity of the pathway. Also visible in figure 3.1 is the action of cytokine-inducible SH2-containing protein (CIS), which is similar to SOCS, and induced in the same manner, this protein sterically blocks the binding of STAT to the cytokine receptor, preventing its activation.

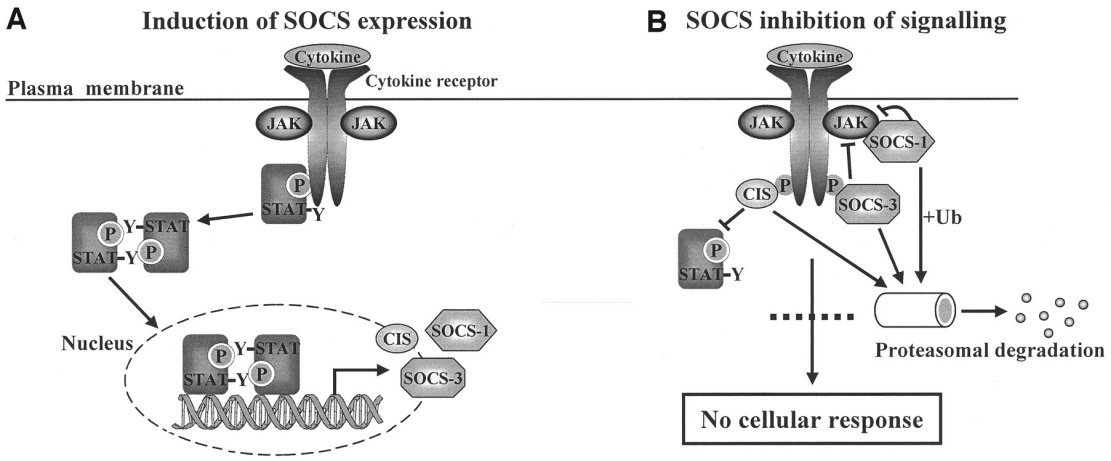

Figure 3.1. A, induction of SOCS expression by binding of cytokine to its receptor activating the Jak/STAT pathway. B, SOCS proteins targeting phosphorylated Jak to inhibit and degrade them as well as CIS blocking the receptor phosphorylation site preventing binding of STAT (adapted from Rønn, Billestrup & Mandrup-Poulsen, 2007).

SOCS have been for a long time considered to have a positive impact on the pathogenesis of DM (Rønn, Billestrup & Mandrup-Poulsen, 2007). Both its action in reducing insulin resistance (Rui et al., 2002) and its role in glomerulopathy (Zhou et al., 2014) has been explored. SOCS are essentially Jak/STAT blockers that occur as a natural part of the cells machinery, making them similar to Jakinibs in that they abrogate signalling through this mechanism and therefore alter the cellular changes Jak/STAT activation invokes. Indeed, it has been shown that enhanced expression of SOCS 1/3 in the kidneys of rats with T1DM improves their renal function (Ortiz-Muñoz et al., 2010).

The phase II clinical trial of the oral Jak1/2 inhibitor, baricitinib reported in a presentation to the American diabetes association's annual meeting in 2015 (Tuttle et al., 2015) that use of this drug reduces urinary ACR. The results of this study are shown below in figure 3.2, showing that there is a dose dependent reduction in the ACR of patients treated with baricitinib. The results of this study indicate that there is efficacy in the use of this class of drugs in patients with DN but no mechanism of action is discussed. The authors to this study also, in another publication (Berthier et al., 2009) state that animal models of DN are not suitable to model this disease correctly.

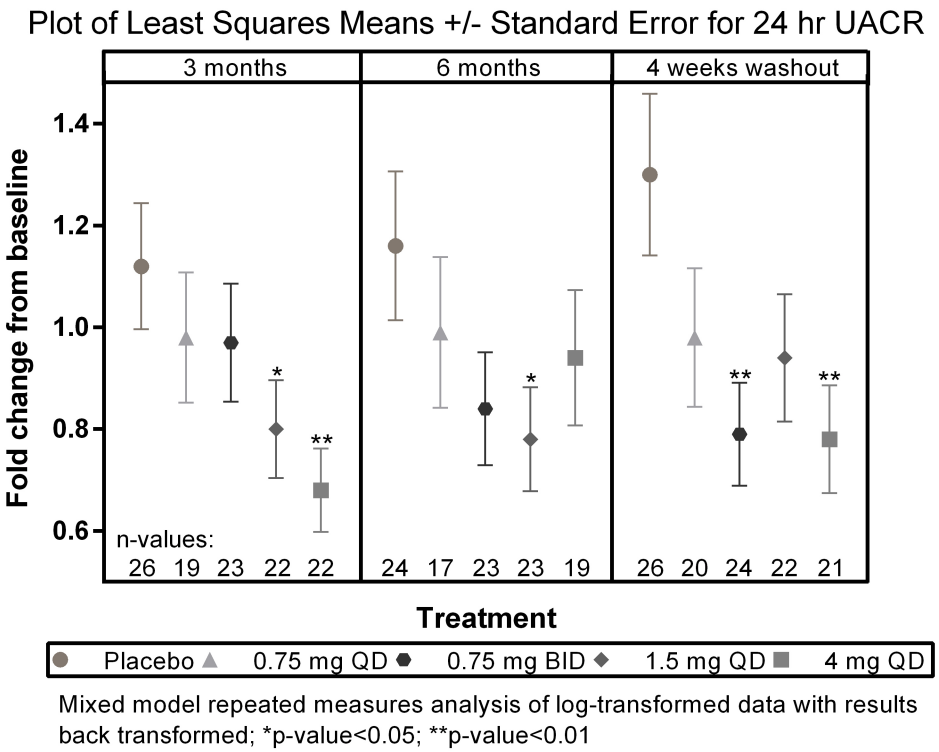

Figure 3.2. Results for Urinary ACR in patients treated with the oral Jak1/3 inhibitor baricitinib in a phase II clinical trial (from Tuttle et al., 2015).

Given the suggested role of Jak/STAT and SOCS in both direct modulation of DN and in the modification of mechanisms of insulin resistance the question is to be posed as to whether the results shown by Tuttle et al., are as a result of a disease modification or a direct effect on the kidney. When we think about animal models of DM that are most appropriate for testing this drug pre-clinically it is important to remember the mode of induction. Most animal models of diabetes currently use a genetic or T1DM-like induced mode of action to adjust insulin signalling rather than the insulin resistance brought on by T2DM. It would seem prudent to explore the mechanism of action of these drugs in T2DM to better understand how we can modulate this pathway in order to bring about better clinical outcomes for these patients and elucidate the true function of these drugs.

In this chapter the oral Jak1/3 inhibitor, baricitinib was administered to animals eating a HFD in order to induce insulin resistance and a T2DM-like phenotype. The effects of this drug on a number of symptoms associated with DN and DM were then assessed. This was in order to begin understanding the mechanism of action of Jakinibs in ameliorating renal injury in patients with DN and to understand whether these drugs are disease modifiers or are directly treating DN.

Materials and Methods

In vivo

Induction of Diabetic Nephropathy

C57BL/6J mice obtained from Charles River UK Ltd. (Margate, Kent) at 9wk of age. Mice were housed with food and water *ad libitum* under regulations and guidance set down in the Animals (Scientific Procedures) Act 1986. Following one week of acclimatisation, mice were randomly divided into three groups.

1. Chow: Housed with access normal mouse chow diet from LabDiet (St. Louis, MO) and water *ad libitum* for 14wk.
2. Vehicle: Housed with access to high-fat diet (HFD) (AIN-76A w/58% fat energy/sucrose/red) from TestDiet (St. Louis, MO) and water *ad libitum* for 14wk. After 6wk on experimental diet, these mice were administered 4% dimethyl sulphoxide (DMSO) vehicle p.o. by oral gavage daily for the remainder of the study.
3. Baricitinib: Housed with access to HFD (AIN-76A w/58% fat energy/sucrose/red) from TestDiet (St. Louis, MO) and water *ad libitum* for 14 wk. After 6wk on experimental diet, these mice were administered 10mg/kg baricitinib in 4% DMSO vehicle p.o. by oral gavage daily for the remainder of the study.

Throughout the duration of the study, mice were measured for their weight and food intake once a week to monitor health and appetite. At completion of the study after 14wk total mice were sacrificed by overdose of ketamine/xylazine and exsanguinated by cardiac puncture before organs were collected for analysis. Blood collected form cardiac puncture was then spun at 3000g, serum was collected and stored at -80°C in aliquots for later analysis. Mice were immediately dissected after cardiac puncture and all organs stored in 10% formalin, RNALater® or at -80°C for later analysis.

Oral Glucose Tolerance Test

Mice were fasted overnight (16h) by transferring to a new clean cage with no access to food but water *ad libitum*. Before beginning protocol the weight of each mouse was established and volume of 15% w/v glucose solution required for a dose of 1.5mg/g calculated. Fasted blood glucose was measured before glucose was administered by oral gavage. Blood glucose was measured at T=10min, 30min, 45min, 60min and 90min. Blood glucose measurement was carried out by removing a very small 1mm section of the end of the tail with scissors allowing collection of a small drop of blood. Blood removed from the tail was tested using a glucosemeter (OneTouch®-Ultra, LifeScan) with glucose test strips (Accu-Chek®, Roche).

Insulin Tolerance Test

Fasting for 4h was performed to normalise insulin levels prior to initiation of the test. Mice were weighed and a volume of insulin for a dose of 0.75U/kg established. Baseline blood glucose measurements were then made and the insulin administered i.p. Blood glucose measurements were taken at 10, 30, 45, 60, 90 and 120min post administration of insulin.

Mouse Model of Ischaemia Reperfusion Injury

C57BL/6J mice obtained from Charles River UK Ltd. (Margate, Kent) at 9wk of age. Surgery was carried out under sterile conditions and temperature was maintained at 37°C throughout. Mice were anaesthetised using isoflurane, abdominal hair removed and injected with 0.3mg/kg buprenorphine s.c. A full midline laparotomy was performed and the kidneys exposed. Renal pedicles were isolated and clamped using non-traumatic microvascular clamps for 30min. Following reperfusion the cavity was closed using proline suture and administered 0.5ml saline i.p. Mice were recovered and returned to their cages with water and food *ad libitum*. 24h after surgery, mice were sacrificed by overdose of ketamine/xylazine and exsanguinated by cardiac puncture.

Ex vivo

Serum Biochemistry

Serum biochemistry was carried out by Dr Keith Burling of the Core Biochemical Assay Laboratory (Cambridge, UK) using the Siemens Dimension EXL autoanalyser.

Urine Biochemistry

Urine biochemistry was partly carried out by Dr Keith Burling of the Core Biochemical Assay Laboratory (Cambridge, UK) using the Siemens Dimension EXL autoanalyser. Urine albumin was established by Dr Keith Burling of the Core Biochemical Assay Laboratory (Cambridge, UK) using a microalbumin ELISA from Bethyl (Montgomery, TX) following the manufacturers guidelines.

Tissue Lysis Protocol

Tissue for analysis was lysed using ice-cold radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors in a bead lyser. Tissues were lysed with a 3:1 w/v ratio of lysis buffer to tissue to standardise protein concentrations. After homogenisation, lysates were centrifuged at 3000g for 5min and supernatant collected with both supernatant and pellet stored at -20°C for later analysis.

Tissue Analysis

All tissue analysis was carried out using ELISA kits (mouse 10-plex pro-inflammatory panel 1) from MSD LLC. (Rockville, MD, USA) following the protocol set down by the manufacturer. Assays were carried out on supernatant of lysate and serum collected at terminal dissection.

Histological Analysis

Histological analysis was made on 4μm samples of tissue that had been fixed in 10% formalin for 24h and embedded in paraffin. Slides were deparaffinised in xylene, rehydrated and stained with Mayers's H&E or Periodic acid Schiff (PAS) and dehydrated before having a coverslip applied with DPX mountant. These slides were viewed using a Nanozoomer digital pathology scanner (Hamamatsu Photonics K.K., Japan). Glomerular and Bowman's capsule sizes were assessed using the Nanozoomer NDP viewer software (Hamamatsu Photonics K.K., Japan). Two cross-sectional measurements (at 90° to one another) were taken for 20 renal corpuscles and averaged before an area for each was established using the formula $A=\pi r^2$ with 20 representative glomeruli being used per mouse.

Periodic Acid Schiff staining was also performed on different sections of the same samples. This was viewed using the same protocol as H&E sections and analysed quantitatively for extra-cellular matrix expansion.

RNA Extraction

Samples of organs stored in RNALater® had RNA extracted using commercially available RNEasy spin columns from Qaigen (Hilden, Germany) following their procedure with the RNA stored at -80°C for later analysis.

cDNA Synthesis

cDNA synthesis for analysis by q-PCR was carried out using QuantiTect® RT kit from Qiagen (Hilden, Germany) following instructions laid out by the manufacturer. The cDNA was stored at -20°C for later analysis with excessive freeze-thaw-cycles avoided.

Quantitive-PCR

cDNA samples synthesised from RNA samples from mouse organs were tested in q-PCR reactions. These reactions were set up in 96-well plates using TaqMan® Fast universal PCR master mix (ThermoFisher, MA, USA). Probes were VIC-labelled, MGB-quenched GAPDH probe for control housekeeping gene and FAM-labelled, MGB-quenched gene of interest probes. Protocol was run using One Step Plus q-PCR machine from Applied Biosystems and data returned in the form of raw C_t values that were internally normalised twice against GAPDH and control sample to produce $2\Delta\Delta C_t$ values as presented.

In vitro

Tissue Culture Technique

Immortalised human podocyte cells were obtained from the laboratory of Prof. Moin Saleem of the University of Bristol. These cells were cultured in RPMI medium (Thermo, UK) with 10% Fetal Bovine Serum (FBS) (ThermoFisher, MA, USA), 5% penicillin/streptomycin (pen/strep) (ThermoFisher, MA, USA) and 5% Insulin, Transferrin and Selenium (ITS) (ThermoFisher, MA, USA). Cells were grown to 80-90% confluence at 32°C and then transferred to an incubator at 37°C for a period of 2wk to differentiate. These cells had a heat activated 'T-antigen' allowing them to grow undifferentiated at 32°C before being differentiated at 37°C at which point the cease to multiply any further and express podocyte specific markers. All cells were used for experiments within 3 days of reaching full differentiation. Cells had a medium change 2h before human recombinant (hr)IL-6 was 'spiked in' to a concentration of 100nM with baricitinib in 0.1% DMSO vehicle and incubated for 6h. Cells were lysed for protein analysis using ice-cold RIPA buffer with protease and phosphatase inhibitors. After homogenisation, lysates were centrifuged at 3000g for 5min and supernatant collected with both supernatant and pellet stored at -20°C for later analysis.

Western Blotting

Protein samples were analysed using western blotting. Samples protein concentration was measured using a standard BCA assay (ThermoFisher, MA, USA) and diluted to a concentration of 1mg/ml total protein. Samples were denatured and reduced with lithium dodecyl sulphate (LDS) loading buffer (ThermoFisher, MA, USA) plus reducing agent (ThermoFisher, MA, USA) at 70°C for 10min. 10µg of protein was added to each well of a sodium dodecyl sulphate-polyacrylamide gel electrophoresis 4-12% Bis-Tris gel (ThermoFisher, MA, USA) and run until the protein front reached the bottom of the gel.

Proteins were transferred to nitrocellulose membranes using an iBlot system from (ThermoFisher, MA, USA). Membranes were then probed using antibodies from cell signalling technologies (CST) (Danvers, MA, USA) with phospho/total parings from different host animals. Fluorescent secondary antibodies from LiCor (Lincoln, NE, USA) were used, with different wavelengths for phospho/total proteins when needed, these membranes were then visualised using a LiCor Odyssey scanner (Lincoln, NE, USA). Analysis was made using ImageStudio software from LiCor (Lincoln, NE, USA).

Materials

All reagents were acquired from Sigma-Adrich Ltd. (Milan, Italy) unless otherwise stated.

Statistical Analysis

All values described are presented as mean ± SEM for n replicates. Statistical validation and exclusion testing was performed using SPSS from IBM (Armonk, NY). Area under the curve (AUC) analysis was carried out using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA). One-way ANOVA with Bonferroni's post-hoc test was carried out on data sets using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA) and a *P*-value of ≤0.05 was considered to be significant. Also used was two-way repeated measures (RM) ANOVA with Bonferroni's post-hoc test using using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA) and a *P*-value of ≤0.05 was considered to be significant.

Results

The Diabetic Condition

Oral Glucose Tolerance Test

Animals treated with vehicle and baricitinib both sustained significantly higher blood glucose measurements throughout the later time points of this test (figure 3.3.A). The higher blood glucose for vehicle and baricitinib mice was significant against chow from 45min onwards. There was no significance between any groups at 10 and 30 min time points. There was no significance between groups based on AUC analysis (figure 3.3.B), though vehicle and baricitinib mice are clearly higher than chow.

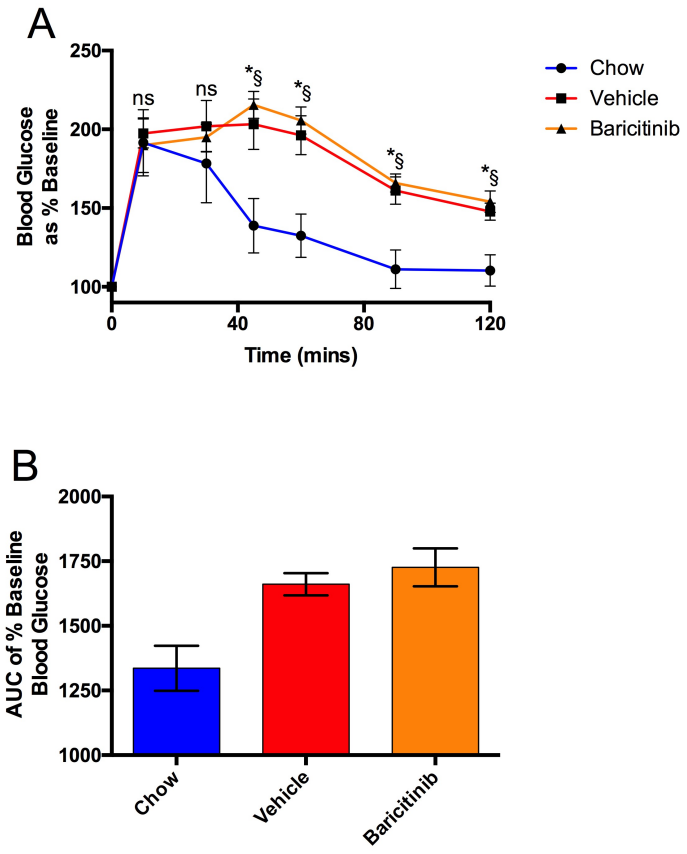

Figure 3.3. A, Time progression for blood glucose during OGTT as percentage of baseline for control, chow animals, diabetic animals treated with vehicle and diabetic animals treated with baricitinib. * P≤0.05 Chow vs. Vehicle, § P≤0.05 Chow vs. baricitinib when analysed by two-way RM-ANOVA. B, AUC analysis for OGTT in A. ★ P≤0.05 vs. Chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Insulin Tolerance Test

No significance was found between any groups at any time points in this test (figure 3.4.A). AUC analysis (figure 3.4.B) shows that mice in the vehicle and baricitinib groups had a significantly lower AUC than chow mice with no significance between vehicle and baricitinib mice.

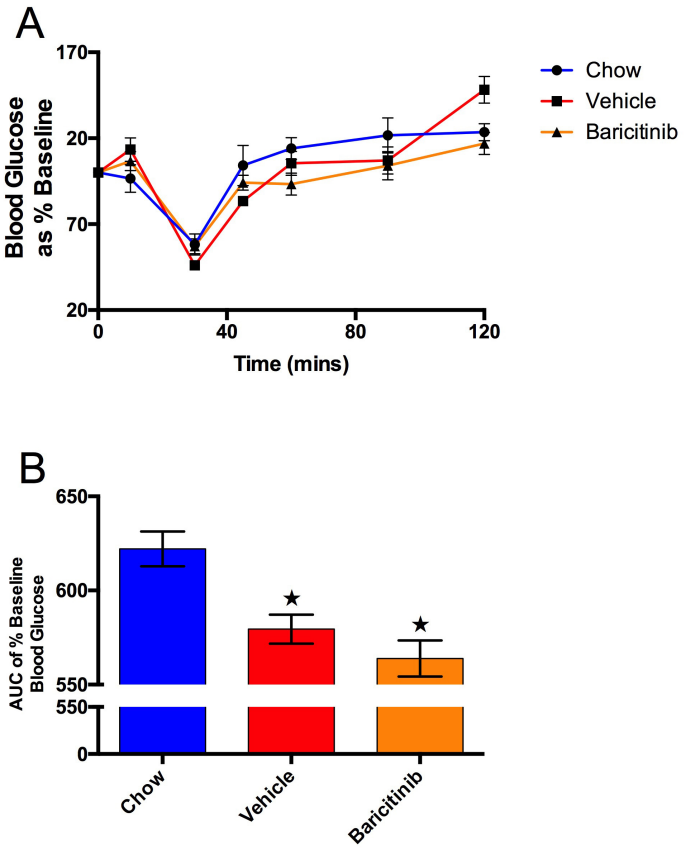

Figure 3.4. Insulin tolerance Test results for blood glucose as percentage of baseline for control, chow animals, diabetic animals treated with vehicle and diabetic animals treated with baricitinib. B, AUC analysis for OGTT in A. ★ P≤0.05 vs. Chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Renal Pathology

Renal Function

No significance was found between the serum creatinine of any groups in figure 3.6.A. Figure 3.6.B shows a small but significant decrease in serum urea in baricitinib mice versus chow with no significance between chow and vehicle or vehicle and baricitinib. Urinary ACR (figure 3.6.C) is significantly elevated in vehicle and baricitinib mice against chow. Mice in the baricitinib group were found to have a significantly lower ACR than vehicle but still significantly elevated against chow.

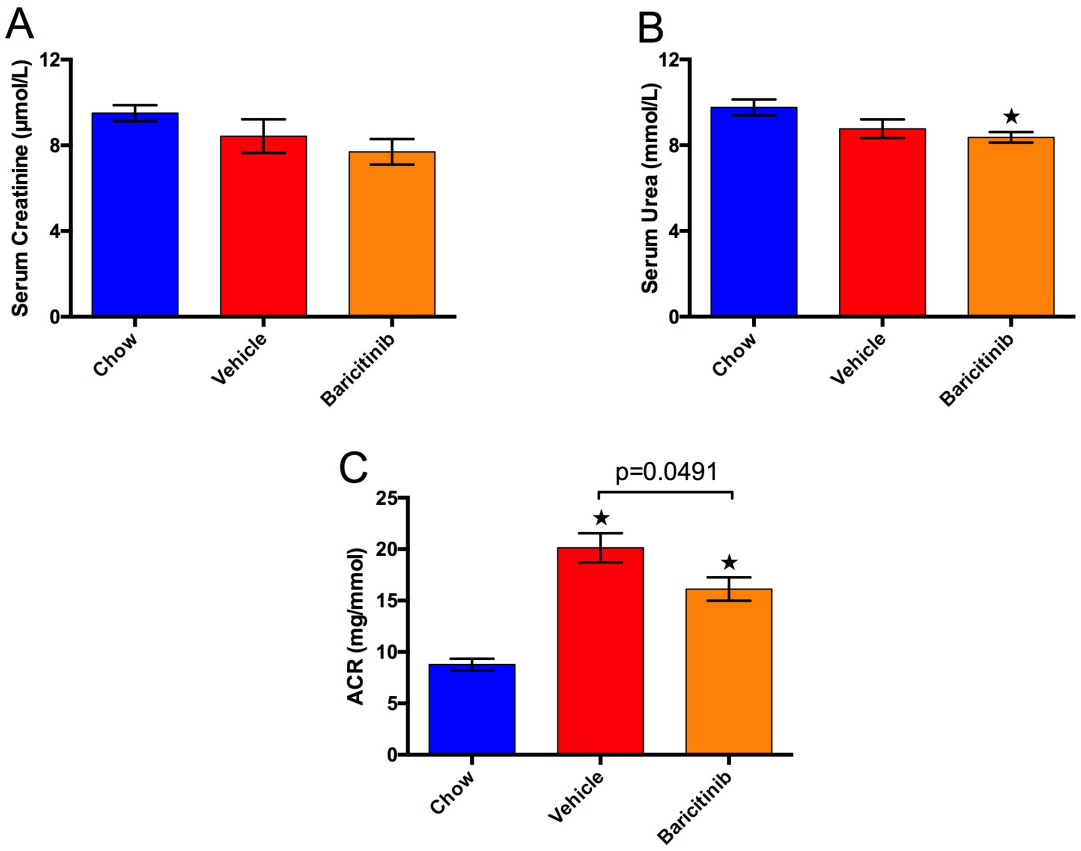

Figure 3.6. A, serum creatinine for mice fed chow or HFD and treated with either vehicle or baricitinib. B, Serum urea. C, urinary ACR. ★ P≤0.05 vs. Chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Renal Histology

Representative images of the renal corpuscles analysed are shown in figure 3.7. It can be seen in figure 3.7 that there is an increase in glomerular area and expansion of the Bowman's capsule in the vehicle group (figure 3.7.B) when compared to chow mice (figure 3.7.A), this increase in these parameters is not seen in baricitinib treated animals (figure 3.7.C). Analysis of the area of the glomerulus (figure 3.8.A) showed that there was a significant increase in area in vehicle treated mice versus both chow and baricitinib with no significance between chow and baricitinib. Bowman's capsule area (figure 3.8.B) showed that there was a significant increase in the area in vehicle mice versus chow and baricitinib with no difference between chow and baricitinib. When the area of the glomerulus was established as a percentage of total corpuscle area (figure 3.8.C) it was found that this was significantly decreased in vehicle treated mice versus both chow and baricitinib with no difference between chow and baricitinib.

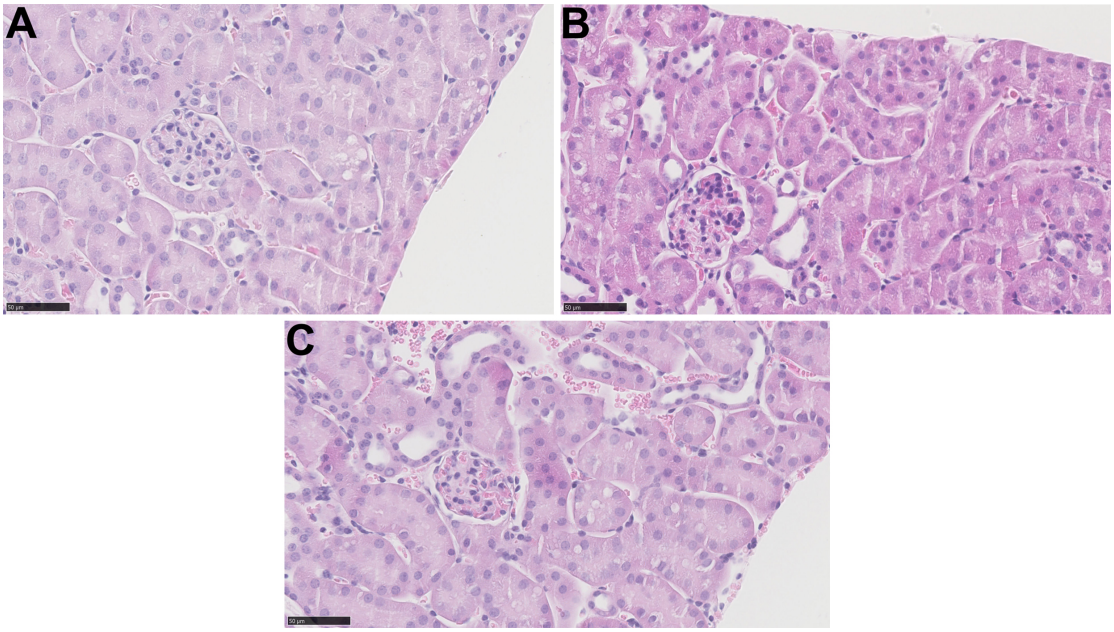

Figure 3.7. Representative images form H&E staining of glomeruli that were used for analysis of glomerular hypertrophy and Bowman's capsule dilation. A, chow. B, vehicle. C, baricitinib. Scale bar is 50µm.

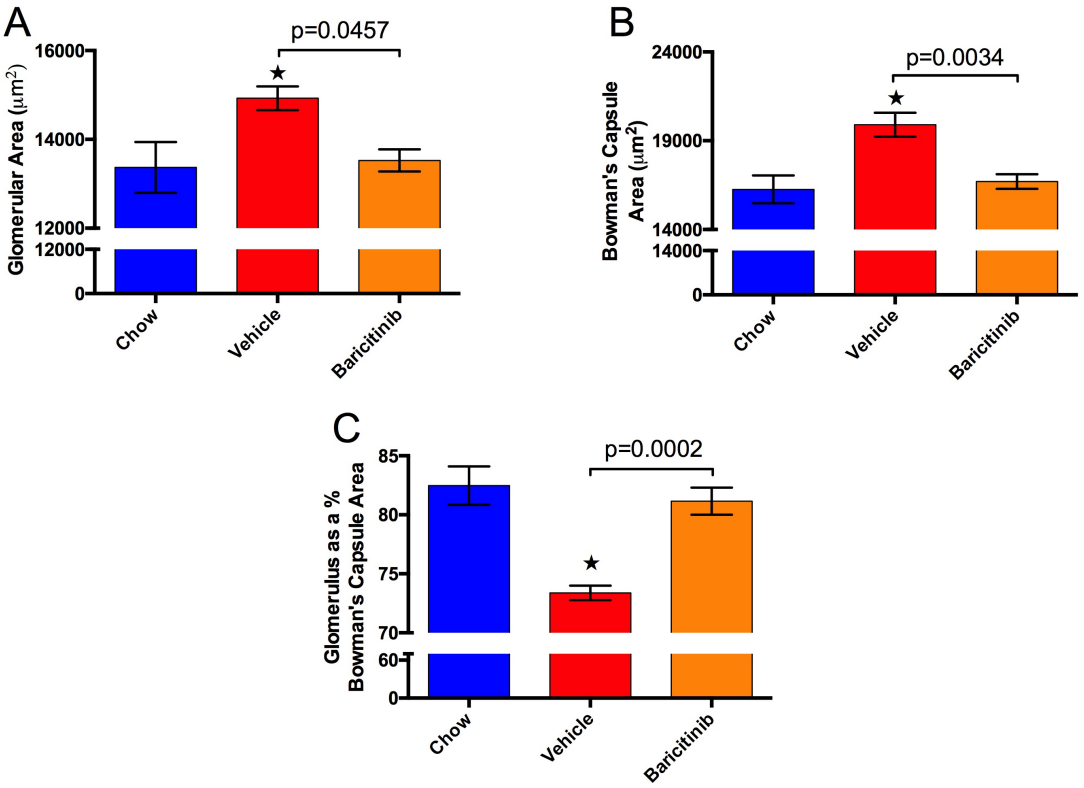

Figure 3.8. Analysis of renal histology from mice fed chow or HFD and treated with either vehicle or baricitinib. A, Glomerular area. B, Bowman's Capsule Area. C, per cent comparison of glomerular area to bowman's capsule area. ★ P≤0.05 vs. Chow when analysed by one-way ANOVA. 'Biological n': Chow: n=8. Vehicle: n=9. Baricitinib: n=10. 'Datapoint n': Chow: n=160. Vehicle: n=180. Baricitinib: n=200 Data presented as mean ± SEM.

Representative images of the glomeruli qualitatively analysed are shown in figure 3.9, here it can clearly be seen that there is an increase in PAS staining in the vehicle group (figure 3.9.B) when compared to chow (figure 3.9.A) and baricitinib treated mice (figure 3.9.C). No clear differences between PAS staining in chow and baricitinib mice was observed.

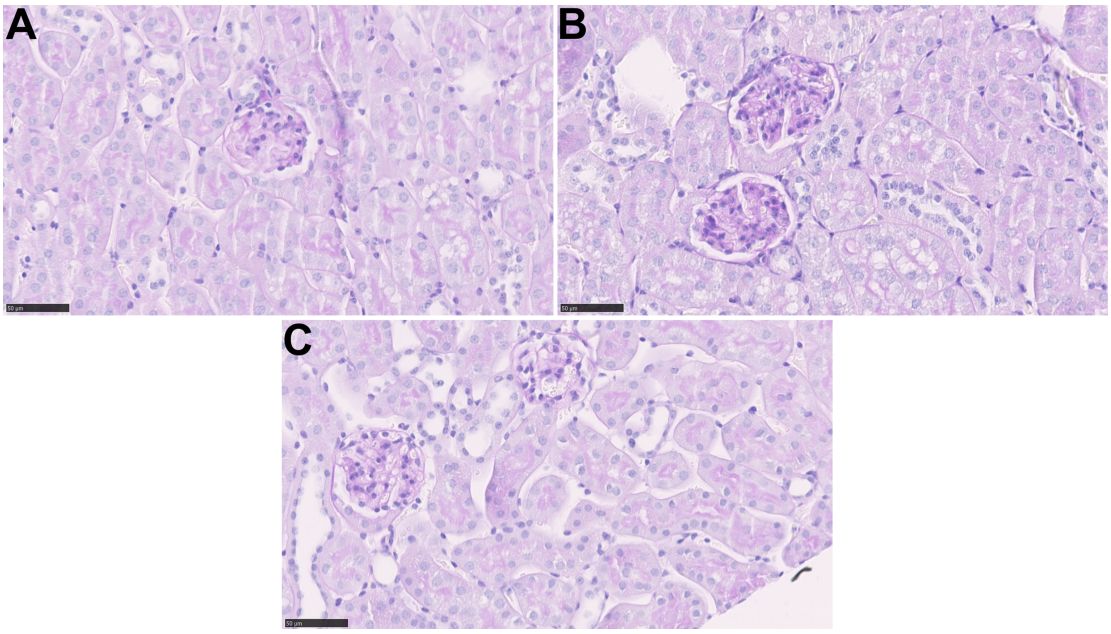

Figure 3.9. Representative images of PAS stained kidney samples from animals fed chow (A), or HFD (B&C) and treated with vehicle (B) or baricitinib (C). Extra-cellular matrix stains dark pink under this stain. Scale bar is 50µm.

Renal Gene Expression

Renal expression of Mafb (figure 3.10.A) was significantly elevated in the vehicle group against chow and baricitinib with no significance between chow and baricitinib. Expression of SOCS2 (figure 3.10.B) was significantly elevated in vehicle mice against both chow and baricitinib mice. There was no significance found between chow and baricitinib groups for SOCS2 expression.

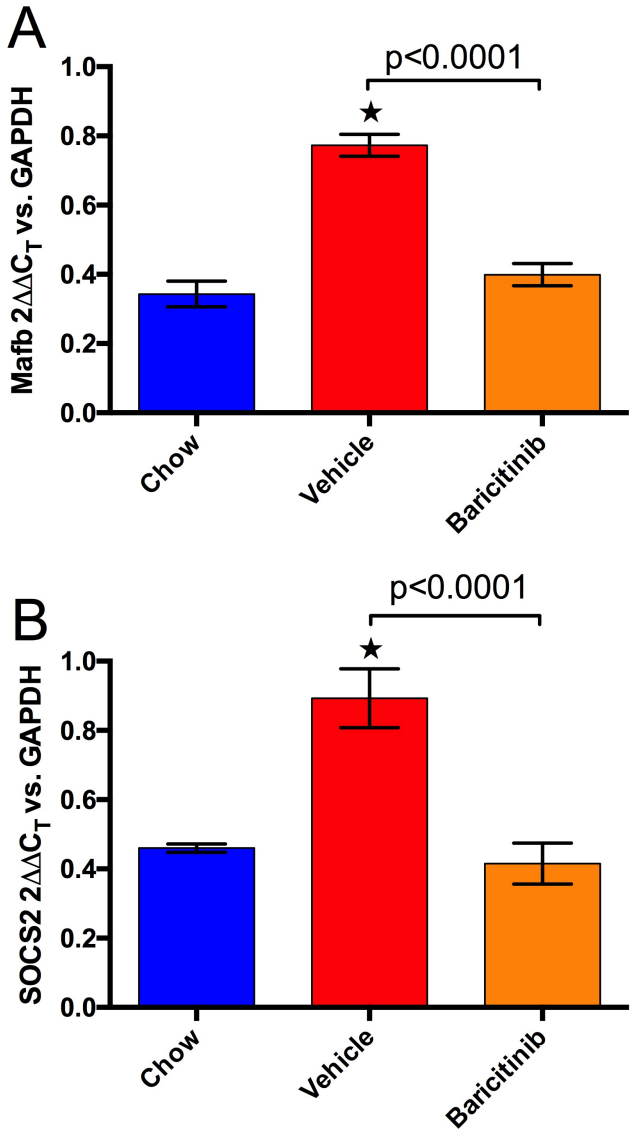

Figure 3.10. A, expression of Mafb gene normalised against GAPDH endogenous control in kidneys of mice fed chow or HFD and treated with either vehicle or baricitinib. B, expression of SOCS2 gene. ★ P≤0.05 vs. Chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Renal Inflammation

No significant changes in KC (figure 3.11.B), IL-1β (figure 3.11.D) and IL-2 (figure 3.11.E) were observed between any groups. TNFα (figure 3.11.A) showed a significant increase in vehicle mice against both chow and baricitinib with no difference between chow and baricitinib groups. IL-10 (figure 3.11.C) was significantly decreased in vehicle and baricitinib against chow with no significance between vehicle and baricitinib.

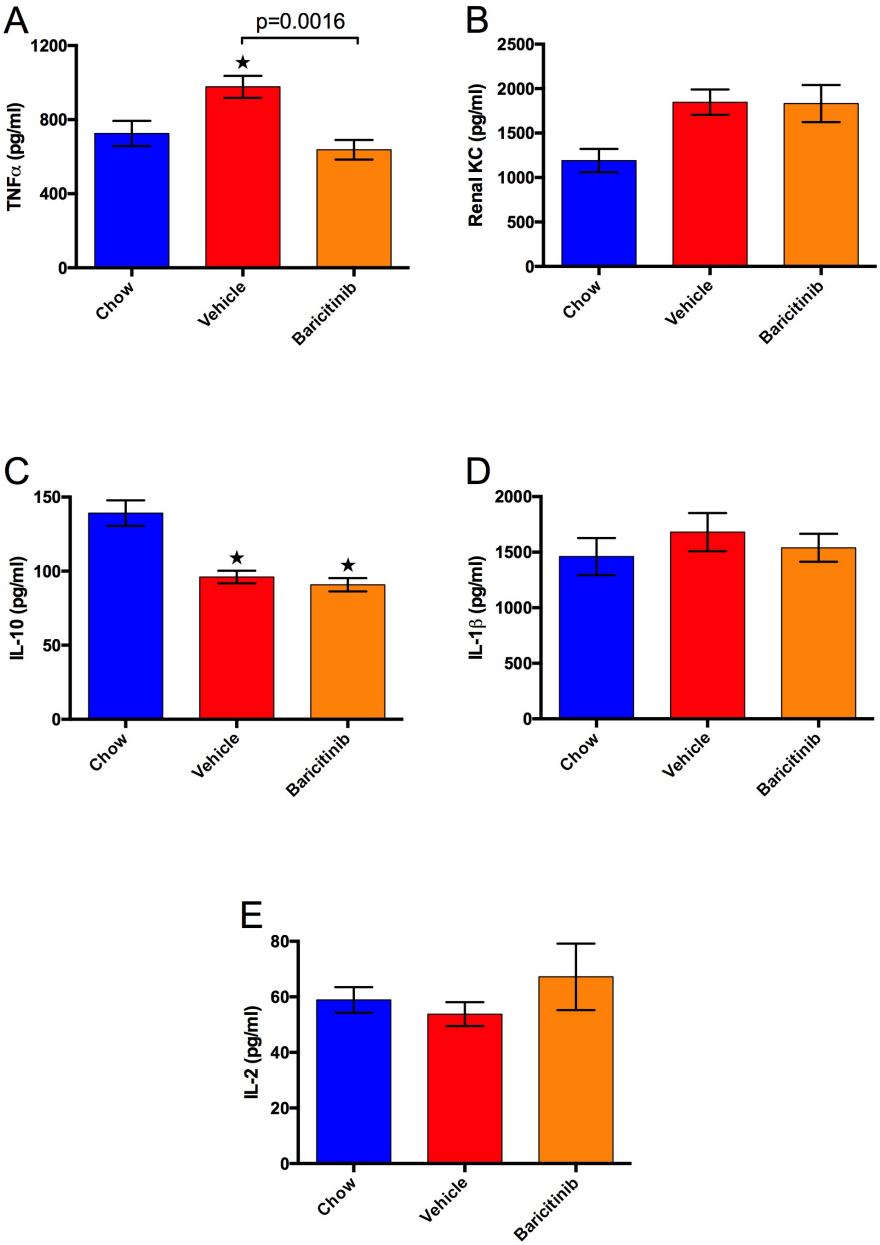

Figure 3.11. Pro-Inflammatory cytokines levels from normalised kidney lysate for mice fed chow or HFD and treated with either vehicle or baricitinib. ★ P≤0.05 vs. Chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=8. Baricitinib: n=8. Data presented as mean ± SEM.

Cell Cycle Regulation

AKT levels (figure 3.12.A) were significantly increased in vehicle treated animals versus both chow and baricitinib, which showed no change against one another. p53 (figure 3.12.B) had no significance between any groups though it was found to be higher in vehicle treated animals with no clear difference between chow and baricitinib. $p21^{cip1/waf1}$ (figure 3.12.C) was significantly elevated in animals treated with vehicle versus both chow and baricitinib groups, which showed no significance between each other. Bax (figure 3.12.D) was significantly elevated in animals treated with vehicle versus both chow and baricitinib, which showed no significance between each other.

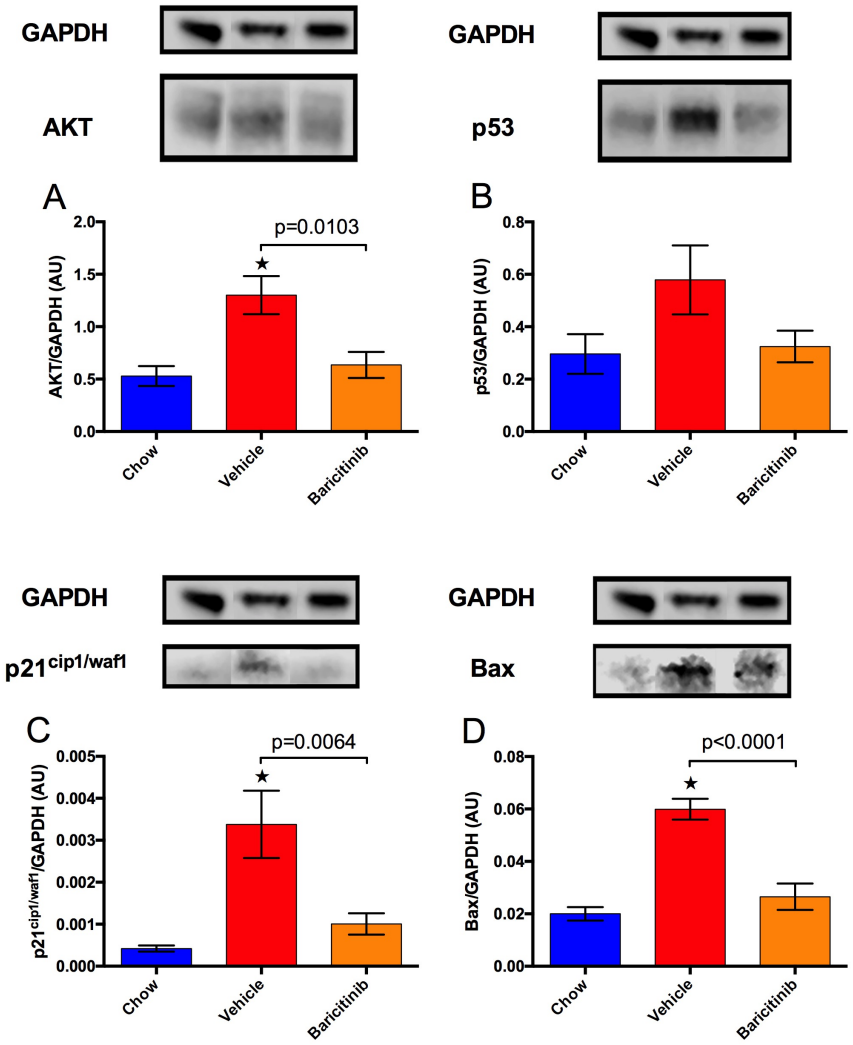

Figure 3.12. Western blot analysis of kidney tissue lysate, relative band intensity versus GAPDH. ★ P≤0.05 vs. Chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Cell Culture Experiments

In cell culture experiments in human podocytes stimulated with hrIL-6, AKT was found to be elevated in these cells when using baricitinib compared with vehicle where no response was seen (figure .A). $p21^{cip1/waf1}$ was unchanged in these cells regardless of the administration of baricitinib (figure 3.13.B).

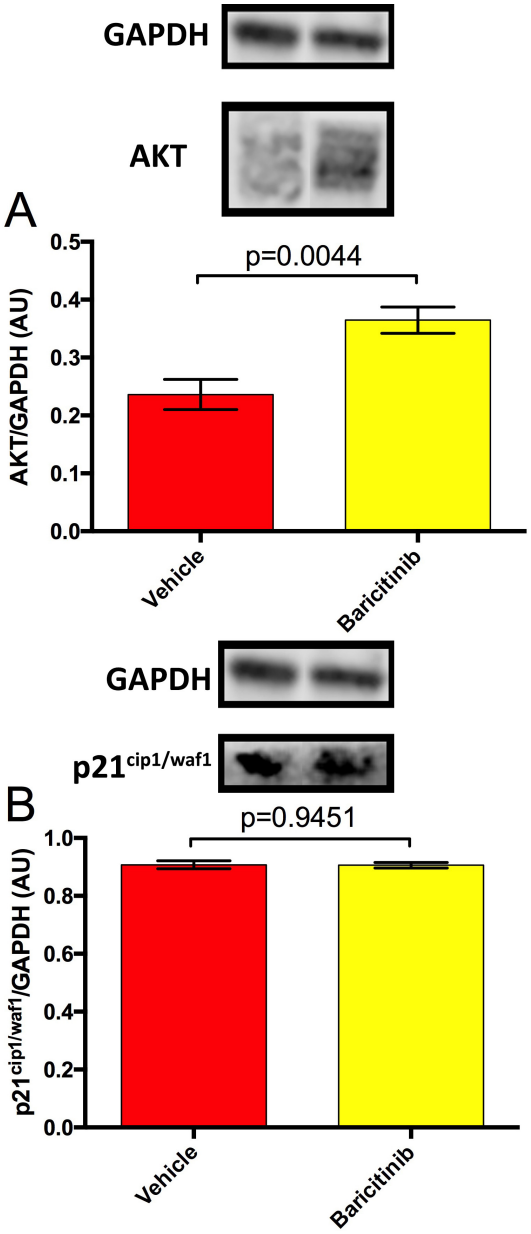

Figure 3.13. $p21^{cip1/waf1}$ and AKT expression in cell culture experiments using $LY8^{+}$ human podocyte cells. Data presented as mean ± SEM.

Ischaemia/Reperfusion Model

Baricitinib and vehicle treated animals had significantly higher urea (figure 3.14.A) and creatinine (figure 3.14.B) versus sham animals. Baricitinib and vehicle groups showed no significance between each other for both creatinine and urea.

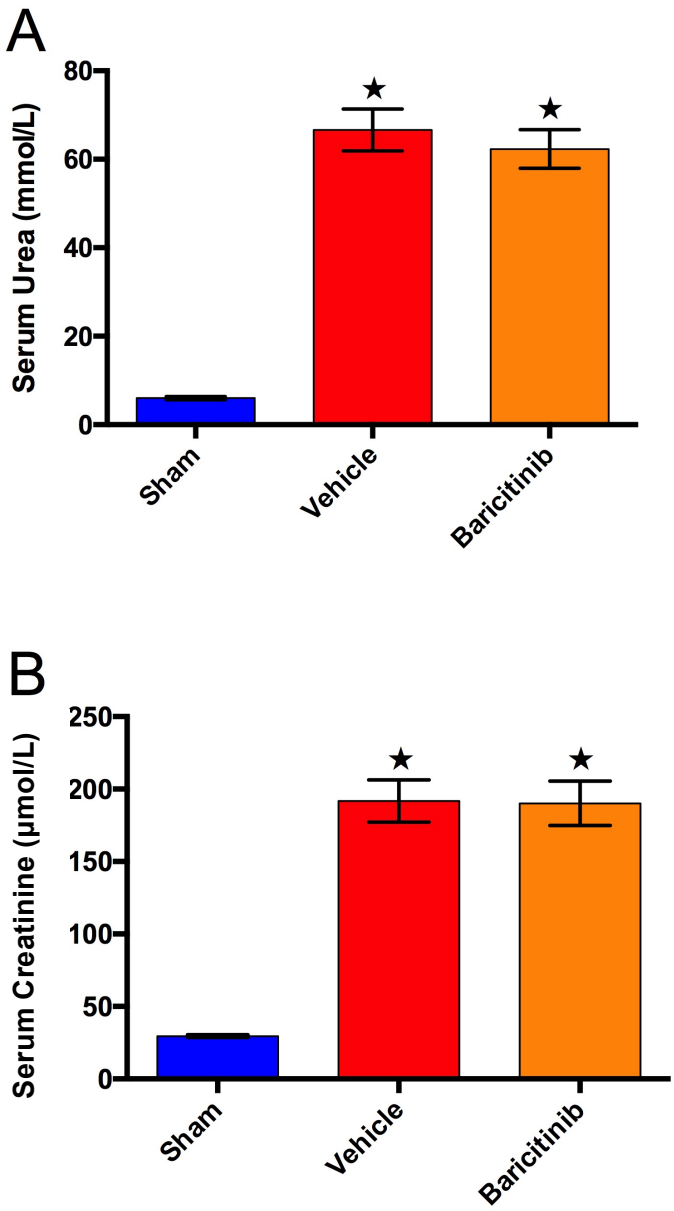

Figure 3.14. Serum urea (A) and creatinine (B) results for animals subjected to renal ischaemia-reperfusion treated with baricitinib or 4% DMSO Vehicle or sham operation. ★ P≤0.05 vs. Sham when analysed by one-way ANOVA. Sham: n=10. Vehicle: n=10. Baricitinib: n=10. Data presented as mean ± SEM.

Discussion

The drug baricitinib has been suggested, and shown to be beneficial in reducing the ACR of patients with diabetes. The question, still remains as to what exactly its mechanism of action is in reducing the symptoms associated with DN. In relation to this, the question of whether it is a generalised anti-inflammatory or works by some other mechanism is yet to be answered. It seems logical that a pathway involved in the transduction of cytokine signals could be considered inflammatory, especially when these signals induce processes such as lymphopoiesis and T-cell activation but the ubiquitous expression of the mechanism means that effects could be far more wide ranging than simply immune activation. The Jak/STAT pathway and its activation have long been seen as a key part of the link between cancer and inflammation (Yu, Pardoll & Jove, 2009). They have an important role to play in the control cell cycle signals from growth factors and in response to cytokine signals (Bhunia et al., 2002). In addition to this it is important to bear in mind that the Jak/STAT pathway also transduces signals from the binding of IL-10, which is often considered to be an anti-inflammatory cytokine (Riley et al., 1999).

Considering the results presented here, firstly we can clearly see that there is a reduction in the diabetes associated renal injury in the mice treated with baricitinib as the ACR of these animals is significantly reduced against vehicle controls. Serum renal markers are also reduced in drug treated animals but it is not raised in animals treated with vehicle so this may be by another mechanism or not clinically significant, and given the mild change, the latter may well be the case. This result shows that this model did indeed induce a diabetic nephropathy-like syndrome in animals fed with HFD. Moreover, these results confirm that, in spite of the reservations of Berthier et al., (2009) that animal models are not suitable for modelling the effect of Jak/STAT inhibition in DN, baricitinib in an animal model does replicate the effects seen in humans by Tuttle et al., (2015).

This model is further vindicated when we carried out tests of insulin sensitivity; both OGTT and ITT showed that there was a significant inhibition of insulin sensitivity with high-fat feeding compared to chow. Interestingly baricitinib administration did not change the insulin sensitivity of these animals, this goes against the findings of Khan et al., (2015) who found that insulin sensitivity was improved with baricitinib in HFD animals. However, in our experiments this lends credence to the argument that this drug's efficacy is not due to a disease modification but a more specific mechanism. Animals fed with HFD and treated with vehicle had an increase in random blood glucose, perhaps analogous with a reading in humans that would prompt father investigations into prediabetes whereas baricitinib did not, this could be a delay in the development of diabetes by baricitinib. It may be concluded from these measurements that animals fed with HFD do indeed develop diabetes but that baricitinib does not affect this condition.

Histological analysis of the terminal nephron from mice in this study shows the classical histological changes associated with DN in animals fed with HFD (Pourghasem, Shafi & Babazadeh, 2015). Animals on HFD showed an increasing size of glomerulus compared with chow animals and an expansion of the Bowman's capsule relative to the glomerulus, indicative of a change in osmotic potential and filtration rate of the glomerulus (Tomlanovich et al., 1987). Expansion in the size of the Bowman's space in the kidney has been linked to changes in glomerular colloid (or oncotic) pressure due to the leakage of albumin into the filtrate changing the osmotic potential of the filtrate comparatively to the blood in the capillaries (Pollak et al., 2014). Baricitinib seems to prevent the dilation of the Bowman's space in this model, perhaps due to the decrease in ACR seen with treatment preventing this, leading us to believe that the kidneys of these animals have greater integrity of their GBM. The increased size of the glomerular apparatus itself is individually indicative of hypertrophy of the mesangium (Fioretto & Mauer, 2007) a classic finding of DN linked to

proteinuria. It appears from these histological findings that there is less expansion of the glomerulus in animals treated with baricitinib, leading us to believe that this is a potential mechanism for this drug's efficacy, reducing mesangial expansion. The conclusion that baricitinib reduces mesangial expansion in mice fed with HFD is shown by PAS staining which can be seen to be less intense in the glomeruli of animals treated with baricitinib.

The drug, baricitinib is used to inhibit the Jak/STAT pathway, whilst doing this; it reduces the expression of SOCS as these are downstream of Jak/STAT activation (Aaronson & Horvath, 2002). It has previously been shown that increases in SOCS expression are beneficial in DN (Zhou et al., 2014). Our study shows that there is an upregulation of this in DN and that this is completely reversed, the beneficial effects of SOCS over expression may well have been due to the suppressive effect these have on the Jak/STAT pathway. In our study, SOCS expression was not elevated in the first place, because the Jak/STAT pathway was inactive due to inhibition by baricitinib.

Mafb expression in the kidneys of animals fed with HFD follows a similar pattern to SOCS; it is elevated under the diseased state compared with sham and reduced with addition of baricitinib. This could indicate something that has not yet been suggested in the literature, that Mafb expression is, at least in part, controlled by the Jak/STAT pathway. Mafb is involved in podocyte differentiation and has been implicated in podocyte effacement (Sadl et al., 2002), a major cause of podocyte loss in many nephropathies (Wagner et al., 2008). It could be, given that it is elevated in the diseased state here that Mafb is upregulated due to Jak/STAT activation and its high level of expression is resulting in differential changes and, therefore, loss of podocyte function which does not occur under Jak/STAT blockade with baricitinib.

When modulation of inflammation is considered as a potential cause of the positive impact that baricitinib has in DN, cytokines tell an interesting and conflicting story. The canonical DM cytokines, IL-1β and TNFα (Salminen, Kaarniranta & Kauppinen, 2012) would be expected to all be significantly elevated. It is therefore counterintuitive that IL-1β is not significantly increased in the kidneys of mice fed with HFD. It is also to be noted that KC, not always associated with DN but has been implicated (Tashiro et al., 2002) and is often associated with acute renal inflammation (Harada et al., 1994). The early phase of DN is where KC is most expected (Tashiro et al., 2002) and this is the stage which we intended on modelling in these animals, so this finding does not support the conclusion that these animals are in early stage DN. IL-10 was found to be depressed in animals fed with HFD, this does paint a picture of a pro-inflammatory environment given the anti-inflammatory properties of IL-10 (Iyer & Cheng, 2012). The only cytokine that was affected by administration of baricitinib was TNFα, this is an interesting finding in that TNFα is chiefly produced by activated macrophages. Macrophages can be activated through the Jak/STAT signalling pathway with cytokines like IL-4 and IL-13 (Gordon, 2003) so it could well be that the increase in macrophage activation came from IL-13 (no data available) which was suppressed by inhibition of the Jak/STAT pathway. It is also interesting that IL-2, highly important in T-cell activation (Suzuki et al., 1995), is not changed in anyway during HFD feeding and Jak/STAT inhibition. T-cells to this point have not been shown to have a strong association with the pathology of DN so it is perhaps unsurprising that this cytokine is not modulated by addition of HFD or administration of baricitinib because these cells are not activated in the first place for them to be affected by this drug.

The profile of inflammatory cytokines in the animals of this study shows that there is a pro-inflammatory component to this disease. However, it is perhaps not what we would expect and certainly not what analysis of human samples has shown (Navarro-González & Mora-

Fernández, 2008). It could be claimed that this model is only of an early stage of DN or perhaps that our model does not fully model the human disease state. When we consider the impact of baricitinib on inflammation in this model it seems that there is little modulation of the cytokine profile and therefore immune and inflammatory activation. It may be that inflammation and inflammatory markers like cytokines are not the best way to measure the effects of this drug and it is in fact the downstream effects of these cytokines that are being altered by the drug.

The next indication that baricitinib has very little impact on inflammation comes from other models of renal inflammation and fibrosis. Far from inhibiting the induction of inflammation, baricitinib administration appears to have little or no impact on the inflammation induced by ischaemia reperfusion injury in our model of AKI. As a model of acute renal inflammation this induces renal inflammation and fibrosis and results in the acute elevation of serum creatinine. Given the efficacy shown by baricitinib in RA, a disease of chronic, rather than acute inflammation it then seems sensible to explore the potential of baricitinib in a more chronic model of renal injury. Data produced within our laboratory by an undergraduate placement student, Vicky Merezhko, under the guidance of myself and other PhD students is shown in figure 3.15. This data indicates that when baricitinib is used in the model of renal fibrosis, UUO, it does not reduce the injury resulting from this model (Merezhko et al., 2016). This is not truly a model of renal inflammation but of fibrosis, so the most common measure of injury is to quantify collagen and fibrin deposits by Picro-Sirius Red (PSR). Collagen and fibrin deposits are an indicator of fibroblast and macrophage activation, which would be expected to be reduced if there was a reduction of renal inflammation, which is not seen when treated with baricitinib. These findings in other models of renal inflammation/fibrosis lead us to believe that baricitinib has very little ability to reduce inflammation induced in common renal diseases.

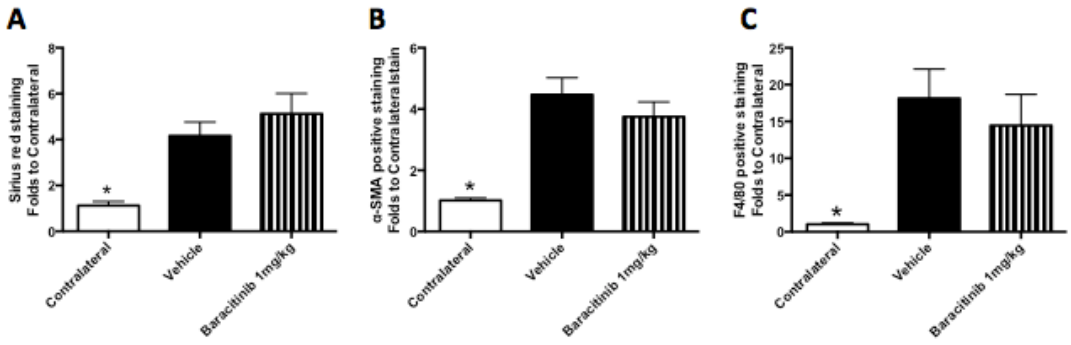

Figure 3.15. Analysis of staining for collagen deposition by PSR staining for animals subjected to UUO treated with vehicle and baricitinib. The deposition of **A.** collagen types I and III stained with Picrosirius red staining **B.** myofibroblasts stained with α-SMA **C.** macrophages stained with F4/80 in murine kidneys following UUO (from Merezhko et al., 2016).

Downstream of Jak/STAT activation are many parts of the machinery for cell cycle modulation. $p21^{cip1/waf1}$ for example plays a pivotal role in cell cycle arrest and induction of apoptosis via the Jak/STAT pathway (Bhunia et al., 2002). We see that $p21^{cip1/waf1}$ is present in highly significant quantities in the kidneys of animals fed HFD and treated with vehicle. Mesangial expansion and overgrowth, leading to occlusion of the glomerular blood vessels is a major driver of the pathology of DN (Steffes et al., 1989) by inducing podocyte apoptosis (Schiffer et al., 2001). It is known that $p21^{cip1/waf1}$ is required for mesangial cell expansion (Al-Douahji et al., 1999, Griffin & Shankland 2004) and that enhanced levels are present in the kidneys of mice subjected to experimental diabetic nephropathy (Kuan, Al-Douahji & Shankland, 1998). When we consider a driver for mesangial expansion in these experiements then $p21^{cip1/waf1}$ would be a clear choice for this, given its highly significant elevation in $p21^{cip1/waf1}$ in the kidneys of animals that have shown significant mesangial expansion.

When we consider the effects of baricitinib in these experiments it is clear that it reduces the expression of $p21^{cip1/waf1}$ in the kidneys of these animals and considering the role which $p21^{cip1/waf1}$ has been shown to play in inducing mesangial expansion, this is a highly likely mechanism of action. It has been shown that $p21^{cip1/waf1}$ inhibition results in a reduction in

the mesangial matrix over-secretion associated with atherosclerosis (Weiss & Randour, 2002), it therefore becomes logical following the work of Bhunia et al. (2002) that inhibition of the Jak/STAT pathway would result in a similar decrease in mesangial expansion in DN. $p21^{cip1/waf1}$ is pro-apoptotic (Levkau et al, 1998, Zhang, Fujita & Tsuruo, 1999), so the results presented here, showing that there is an increase in the expression of genes such as Bax and p53 in vehicle treated mice mirrors this. In these blots, baricitinib appears to decrease the damaging effects of mesangial expansion via p21.

To conclude, the results seen by Tuttle et al., 2015 appear to be reproducible in a mouse model of DN induced by feeding with HFD under our conditions. This 'back-translation' from human studies into mice has enabled us to make some interesting in-roads towards discovering the specific mechanism by which baricitinib makes its positive effects on this disease state. Baricitinib appears to have very little effect on the inflammatory status of the diabetic kidney but does appear to have an impact on the cell cycle of the cells within the kidney. Given the prominent role of the glomerular apparatus in DN and the lack of pathology in animals treated with baricitinib it could well be that the effects of baricitinib are mediated by prevention of mesangial expansion through cell cycle arrest and apoptosis via the p53/p21 pathway. These results however should be treated with caution when the potential renal toxicity shown by a lowered serum urea against controls is considered. The use of this drug at doses such as those in this study in humans has not yet been declared safe so there could still be toxicity that has not yet been found.

Chapter IV: Baricitinib in Treating the Hypercholesterolaemia Associated with Obesity and Diabetes Mellitus

Introduction

Hyperlipidaemia and specifically hypercholesterolaemia is a condition commonly associated with both obesity and DM. This condition is dangerous for patients with DM as it is a contributory factor in the elevated risk of CVD in the population (Haffner et al., 1998) as well as leading to worsening of the diabetic condition itself (Lupi et al., 2002, Khan et al., 2015). Treatment for these is normally associated with conventional cholesterol-lowering medication, such as statins (NICE, 2016). Drug treatments targeted at the underlying mechanisms of diabetic hypercholesterolaemia specifically are not yet in clinic and due to the effectiveness of statins in lowering the lipid profiles of patients with DM, there is not a lot of call for anything specific to the diabetic condition.

The Jak/STAT pathway has had very little implication in any form of hyperlipidaemia thus far. It has however been suggested to be related to the role of adipose inflammation in DM and the part that must play in developing diabetes (Khan et al., 2015). It has further been suggested that amelioration of inflammation in adipose tissues can lead to a reduction in an elevated lipid (specifically cholesterol) profile of mice fed with a HFD (Neyrinck et al., 2013). Moreover, it is shown by Khan et.al (2015) that there is a role of Jak/STAT related inflammation in the induction of adipose related insulin resistance. When these observations are combined, it is not too much of a stretch to believe that Jak/STAT inhibition could lead to positive effects in diabetes induced hypercholesterolaemia.

Modulation of serum lipid levels is made by many mechanisms and is therefore affected by many changes in physiology. As a method of energy transfer within the body, fats and specifically free fatty acids (FFA), their synthesis, degradation, storage and usage are controlled by the hormones insulin and glucagon. Though insulin and glucagon are the major metabolic hormones within the body, their release is heavily influenced by signals from outside. The part that glucose has to play in the release of insulin and glucagon is well known and has been covered in chapter I. More applicable to this chapter however is the role of the gut incretins, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). Gut incretins are released by the endocrine cells of the gut (Mortensen et al., 2003) in response to the ingestion of food. One of the major functions of incretins is to enhance the elevation of the levels of insulin in preparation for the postparandial spike in blood glucose (Turton et al., 1996) as summarised in figure 4.1.

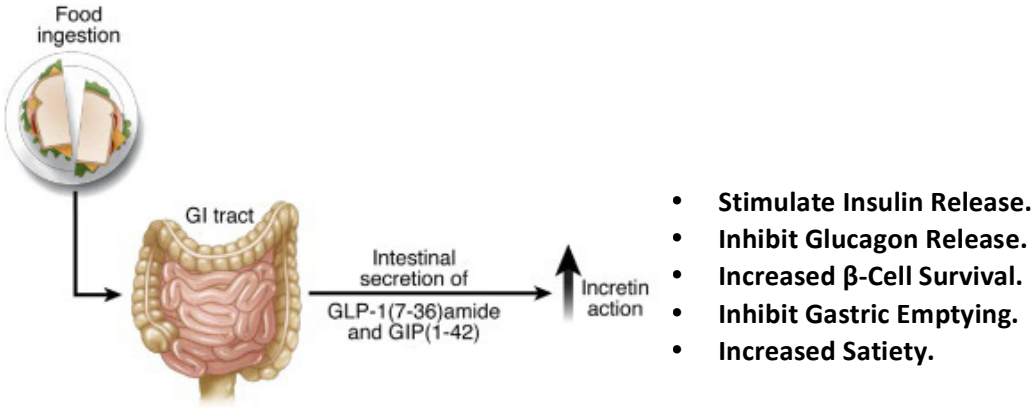

Figure 4.1. The basic action of gut incretins (adapted from Baggio & Drucker, 2007).

Glucagon-like peptide-1 is released from intestinal L-cells in response to glucose and fat intake to the stomach and is produced from alternate splicing of the proglucagon gene as shown in figure 4.2. Figure 4.2 shows that there is an alternate splicing of this gene in the intestine compared to the pancreas resulting in the generation of GLP-1 as opposed to glucagon. Though the proglucagon gene is expressed in the pancreas, the splicing necessary to produce GLP-1 does not occur in the pancreas (Ørkov at. al. 1986).

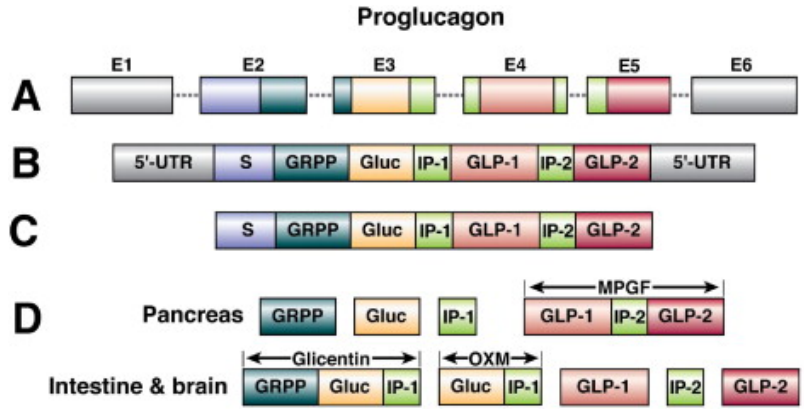

Figure 4.2. Structures of (*A*) the proglucagon gene, (*B*) mRNA, and (*C*) protein. (*D*) Tissue-specific posttranslational processing of proglucagon in the pancreas leads to the generation of Glicentin-related polypeptide (grpp), glucagon (gluc), intervening peptide-1 (ip-1), and major proglucagon fragment (mpgf), whereas glicentin, oxyntomodulin (oxm), intervening peptide-2 (ip-2), and GLP-1 and GLP-2 are liberated after proglucagon processing in the intestine and brain (from Baggio & Drucker, 2007).

Glucagon-like peptide-1 and GIP result in a number of downstream effects throughout the body (figure 4.3) including, increased gastric emptying, a decrease in appetite and importantly for diabetic patients, changes in glucose control. Glucagon-like peptide-1 signals via a G-protein coupled receptor (GPCR) on the surface membranes of both α and β cells in the islets of the pancreas (Baggio & Drucker, 2007). When it binds, GLP-1 prevents the release of glucagon and increases release of insulin.

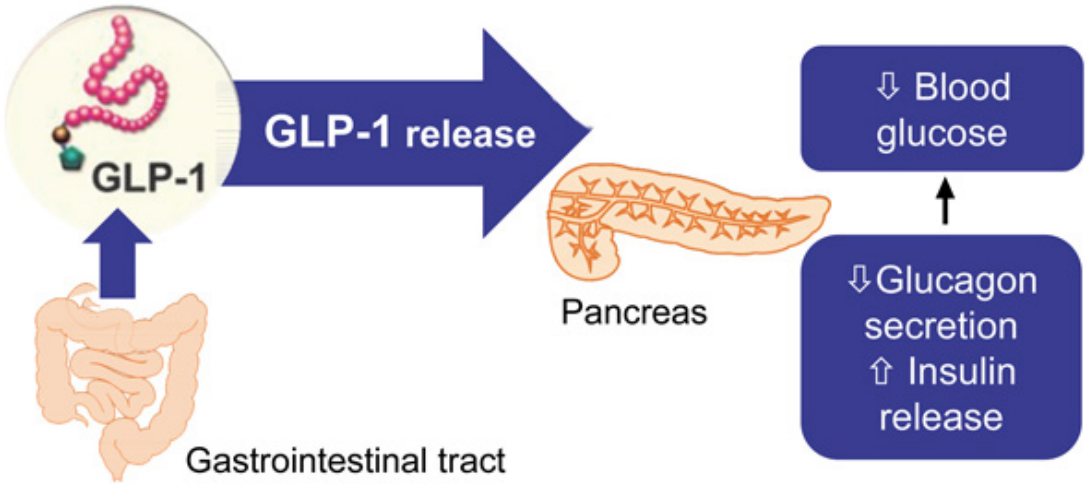

Figure 4.3. Effects of GLP-1 on the body (from Cohen et al., 2013).

Glucagon-like peptide-1 is degraded by dipeptidyl peptidase 4 (DPP-4) in the blood stream (Neumiller, 2008). DPP-4 inhibitors or 'gliptins' are used in diabetes to treat T2DM patients (Pratley and Salsali, 2007). These drugs are intended to improve the reaction of patients with T2DM to insulin by elevating their baseline levels of insulin (Pratley and Salsali, 2007), this is done by preventing the breakdown of GLP-1 into its inactive form as shown in figure 4.1. This inhibition of GLP-1 degradation results in elevated blood GLP-1 levels and therefore constitutively higher insulin and lower glucagon as a result (Barnett, 2006).

Glucagon-like peptide-1 (GLP1) plays a role in gut lipid absorption by promoting its uptake in the intestine (Qin et al., 2005); this clearly has an impact on the serum levels of lipid. Further to absorption modulation, GLP-1 had direct effects on the metabolism of lipids in the adipose tissue (Villanueva-Penacarrillo et al., 2001). Then you also consider that GLP-1 will modulate the levels of glucagon and insulin, it is highly likely that GLP-1 will also exert effects on the serum lipid levels indirectly via these hormones.

The part that inflammation plays in diabetes is partly linked to regulation of metabolic hormones as shown by recent studies by Kahles et al., (2014). Glucagon-like peptide-1 secretion is elevated with IL-6 stimulation of the intestinal L-cells, which are considered to be its primary source (Kahles et al., 2014). IL-6 elicits its effects on the cell via its receptor (IL6ra), which associates with gp130 allowing the binding and phosphorylation of Jak followed by STAT in the normal pathway progression (Zhou L et al., 2007).

These investigations were initially aimed to see in what conditions the Jak/STAT pathway is activated in the kidney to find the best conditions under which it can be inhibited. It was then aimed at recreating, pre-clinically, the conditions of a phase-II clinical trial using a Jak1/3 inhibitor in DN. It was also noted during the assessment of diabetic mice for DN that

there was a change in the lipid profile of the mice treated with the Jak inhibitor, these findings therefore warranted further investigation. In this chapter data and findings from diabetic mice treated with the oral Jak1/3 inhibitor 'baricitinib' are compared with those treated with a vehicle solution.

Materials and Methods

In vivo

Lipopolysaccharide/Peptidoglycan Induced Model of Sepsis/Whole-Body Inflammation

C57BL/6J mice were obtained from Charles River UK Ltd. (Margate, Kent) at 9wk of age. Mice were housed with food and water *ad libitum* under regulations and guidance set down in the Animals (Scientific Procedures) Act 1986. Mice were injected i.p. with cocktail of lipopolysaccharide (LPS) and peptidoglycan (PepG) at a dose of 9/1mg/kg respectively and returned to their cages. After 24h, mice were sacrificed by overdose of Ketamine/Xylazine and exsanguinated by cardiac puncture before organs were collected for analysis.

Unilateral Urethral Obstruction Model of Renal Fibrosis

C57BL/6J mice obtained from Charles River UK Ltd. (Margate, Kent) at 9wk of age. Mice were housed with food and water *ad libitum* under regulations and guidance set down in the Animals (Scientific Procedures) Act 1986. All surgery was carried out under sterile conditions body temperature was maintained at 37°C using a homoeothermic mat and temperature monitor. Mice were anaesthetised using isoflurane, hair removed by hair removal cream and injected with 0.3mg/kg buprenorphine s.c. once full anaesthesia was achieved. A full midline laparotomy was performed and the kidneys exposed. The right urethra was isolated using microdissecting forceps and occluded using mersilk suture twice, once proximally to the kidney and once distally, the abdominal cavity was closed using 3-0 proline suture. Mice were recovered and returned to their cages with water and food *ad libitum*. 7 days after surgery, mice were sacrificed by overdose of ketamine/xylazine and exsanguinated by cardiac puncture before organs were collected for analysis.

Induction of Diabetes Mellitus

C57BL/6J mice obtained from Charles River UK Ltd. (Margate, Kent) at 9wk of age. Mice were housed with food and water *ad libitum* under regulations and guidance set down in the Animals (Scientific Procedures) Act 1986. Following one week of acclimatisation, mice were randomly divided into three groups.

1. Chow: Housed with access normal mouse chow diet from LabDiet (St. Louis, MO) and water *ad libitum* for 14wk.
2. Vehicle: Housed with access to HFD (AIN-76A w/58% fat energy/sucrose/red) from TestDiet (St. Louis, MO) and water *ad libitum* for 14wk. After 6wk on experimental diet, these mice were administered 4% DMSO vehicle p.o. by oral gavage daily for the remainder of the study.
3. Baricitinib: Housed with access to HFD (AIN-76A w/58% fat energy/sucrose/red) from TestDiet (St. Louis, MO) and water *ad libitum* for 14wk. After 6wk on experimental diet, these mice were administered 10mg/kg baricitinib in 4% DMSO vehicle p.o. by oral gavage daily for the remainder of the study.

Throughout the duration of the study, mice were measured for their weight and food intake once a week to monitor health and appetite. At completion of the study after 14wk total mice were sacrificed by overdose of Ketamine/Xylazine and exsanguinated by cardiac puncture before organs were collected for analysis. Blood collected form cardiac puncture was then spun at 3000g, serum was collected and stored at -80°C in aliquots for later analysis. Mice were immediately dissected after cardiac puncture and all organs stored in 10% formalin, RNALater® or at -80°C for later analysis.

Magnetic Resonance Imaging

Mice were anaesthetised using isoflurane and placed on a bed with water heated to 50°C passed through it to maintain body temperature. Monitoring and gaiting of respiration was made using a pressure sensor under the abdomen of the mouse. The mouse was imaged in a Brucker ICON 1T preclinical magnetic resonance imaging (MRI) scanner using the body coil and a T2 weighted rapid imaging with refocused echoes 3D isotropic image with repetition time of 1500ms and echo time of 84ms and a voxel size of 0.219 X 0.375 X 0.375mm.

Images were analysed using the vivoquant software from invicro LLC (Boston, MA, USA). 3D regions-of-interest (ROI) were drawn to isolate the quadriceps muscle of a randomly selected mouse. Within this ROI, a threshold was set for all pixels appearing to contain fat and the volume of those pixels quantified by the software, results for both of these volumes are presented as a percentage of the total quad area exceeding the fat threshold.

Food Intake & Body Weight

Mice were weighed on a balance, at approximately the same time of day, every 7 days from the initiation of diet change to establish their body weight for dosing and weight gain data. At the same time as mouse weight measurement food was also weighed and compare to the previous week with 140g of fresh food replaced into the cages after weighing, this difference in food was then averaged per mouse in that cage.

Ex vivo

Serum Biochemistry

Serum biochemistry was carried out by Dr Keith Burling of the Core Biochemical Assay Laboratory (Cambridge, UK) using the Siemens Dimension EXL autoanalyser.

Tissue Lysis Protocol

Tissue for analysis was lysed using ice-cold RIPA buffer with protease and phosphatase inhibitors in a bead lyser. Tissue concentrations of analyte were normalised for total protein concentration, which was assessed by Peirce BCA assay (ThermoFisher, MA, USA). After homogenisation, lysates were centrifuged at 3000g for 5min and supernatant collected with both supernatant and pellet stored at -20°C for later analysis.

Tissue Analysis

All tissue analysis was carried out using ELISA kits (mouse 10-ples proinflammatory panel 1 or mouse total insulin/glucagon/GLP-1) from MSD LLC. (Rockville, MD, USA) following the protocol set down by the manufacturer. Assays were carried out on supernatant of lysate and serum collected at terminal dissection.

Histological Analysis

Histology was carried out on using sections of snap frozen tissue. Tissues were briefly thawed and fixed using Neutral Buffered Formalin then stained using oil-red-O. Tissues were differentiated using Isopropyl alcohol and counterstained using Mayer's Haematoxylin before being cover-slipped using aqueous mountant. Slides were analysed after being visualised using a Hamamatsu Nanozoomer.

RNA Extraction

Samples of organs stored in RNALater® had RNA extracted using commercially available RNEasy spin columns from Qaigen (Hilden, Germany) following their procedure with the RNA stored at -80°C for later analysis.

cDNA Synthesis

cDNA synthesis for analysis by q-PCR was carried out using QuantiTect® Reverse Transcription (RT) kit from Qiagen (Hilden, Germany) following instructions laid out by the manufacturer. cDNA was stored at -20°C for later analysis with excessive freeze-thaw-cycles avoided.

Quantitive-PCR

cDNA samples synthesised from RNA samples from mouse organs were tested in q-PCR reactions. These reactions were set up in 96-well plates using TaqMan® Fast universal PCR master mix (ThermoFisher, MA, USA). Probes were VIC-labelled, MGB-quenched GAPDH probe for control housekeeping gene and FAM-labelled, MGB-quenched gene of interest probes. Protocol was run using One Step Plus q-PCR machine from Applied Biosystems and data returned in the form of raw C_t values that were internally normalised twice against GAPDH and control sample to produce $2\Delta\Delta C_t$ values as presented.

Alternative Quantitive-PCR Protocol

Pre-loaded RT² Profiler™ PCR Array Mouse JAK / STAT Signaling Pathway 384-well Plates from Qiagen (Hilden, Germany) were used with SYBR Green reagents. This data was anlysed using the Qiagen online RT^2 analyser tool, which uses a panel of housekeeping genes to normalise the expression levels.

Materials

All reagents were acquired from Sigma-Adrich Ltd. (Milan, Italy) unless otherwise stated.

Statistical Analysis

All values described are presented as mean ± SEM for n replicates. Statistical validation and exclusion testing was performed using SPSS from IBM (Armonk, NY). Area under the curve (AUC) analysis was carried out using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA). One-way ANOVA with Bonferroni's post-hoc test was carried out on data sets using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA) and a *P*-value of ≤0.05 was considered to be significant. Also used was two-way RM-ANOVA with Bonferroni's post-hoc test using using GraphPad™ Prism® 6 for mac (GraphPad Software, San Diego, Ca. USA) and a *P*-value of ≤0.05 was considered to be significant.

Results

Regulation of Jak/STAT Associated Genes in Disease Models

Data presented below in figure 4.4 represents the findings of PCR analysis of the kidneys of animals subjected to UUO and LPS and mice aged for 24 months and 3 months, naïve mice. STAT1 (figure 4.4.A), STAT2 (figure 4.4.B) and STAT3 (figure 4.4.C) were all elevated in the LPS model against 3 months mice, but this was only significant in STAT1; no other groups showed significance against 3 months. STAT4 (figure 4.4.D) was significantly elevated in UUO against 3 months naïve mice with no changes for other groups. STAT5a (figure 4.4.E), STAT5b (figure 4.4.F) and STAT6 (figure 4.4.G) showed no significant changes in any groups.

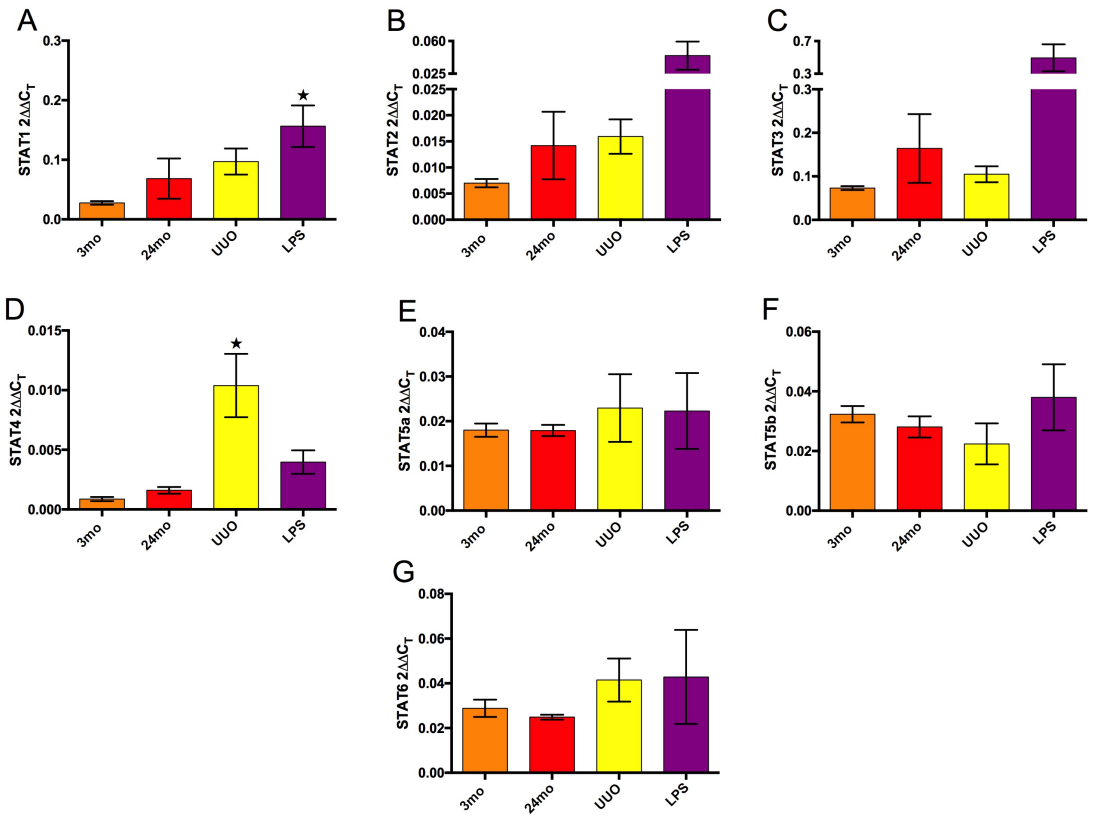

Figure 4.4. Regulation of STAT genes in mouse models of renal disease. ★ P≤0.05 vs. 3mo when analysed by one-way ANOVA. 3mo: n=4. 24mo: n=4. UUO: n=4. LPS: n=4. Data presented as mean ± SEM.

Figure 4.5 shows data for SOCS expression in the same disease states as in figure 4.4. SOCS1 (figure 4.5.A), SOCS2 (figure 4.5.B) and SOCS3 (figure 4.5.C) all showed an increase in expression in the LPS group versus 3 months, though the increase in SOCS1 was non-significant and no other groups showed changes against 3 months. SOCS4 (figure 4.5.D) and SOCS5 (figure 4.5.E) showed no significant changes in their expression across any groups.

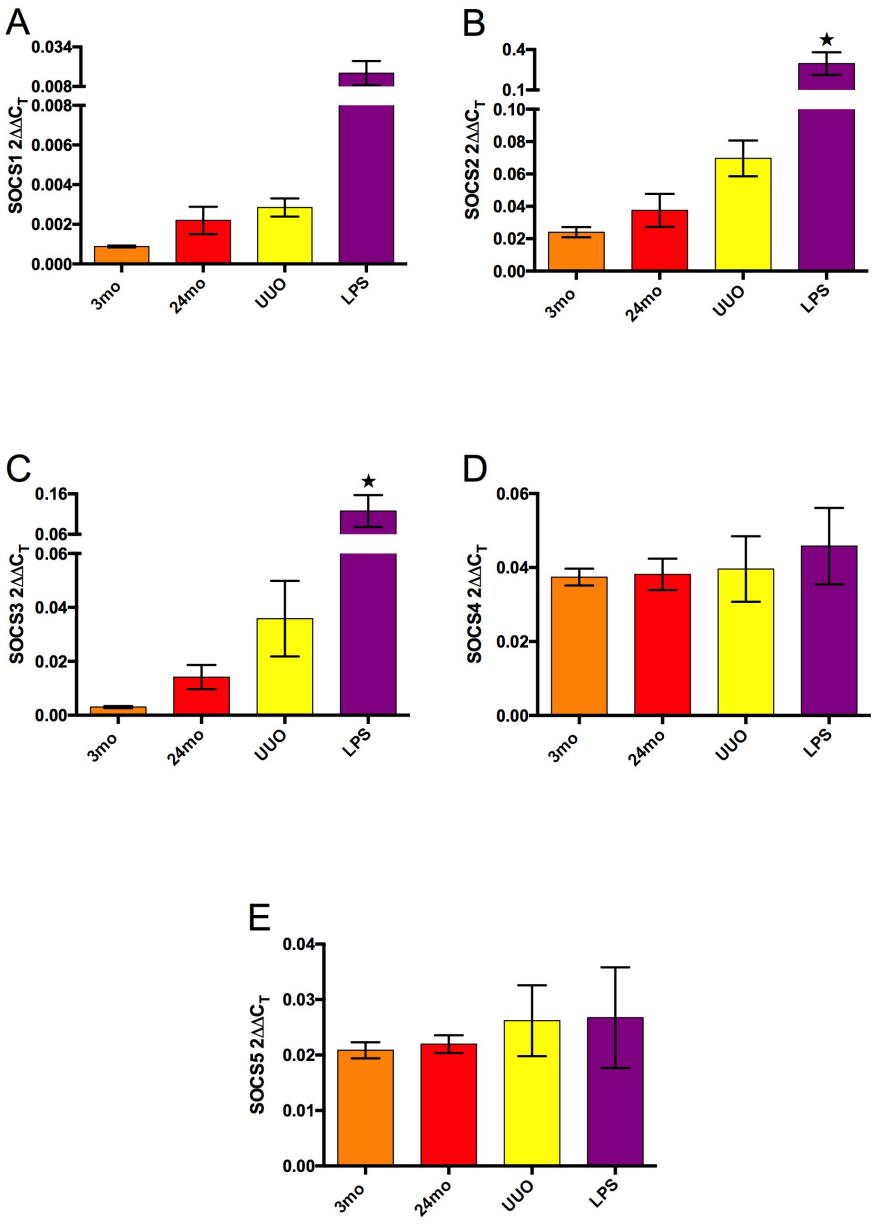

Figure 4.5. Regulation of SOCS genes in the mouse models of renal disease, UUO, LPS treated mice and mice aged for 24mo compared to 3month old naïve mice. ★ P≤0.05 vs. 3mo when analysed by one-way ANOVA. 3mo: n=4. 24mo: n=4. UUO: n=4. LPS: n=4. Data presented as mean ± SEM.

Serum Measurements of Diabetic Animals

Serum Lipids

Both serum total cholesterol (figure 4.6.A) and HDL (figure 4.6.B) showed an increase in both vehicle and baricitinib groups versus chow. Total cholesterol and HDL were found to be significantly lower in baricitinib groups versus vehicle but baricitinib remained significantly elevated against chow. Serum LDL (figure 4.6.C) and HDL: LDL ratio (figure 4.6.D) displayed an increase in vehicle versus chow and baricitinib groups with no significance between chow and baricitinib mice.

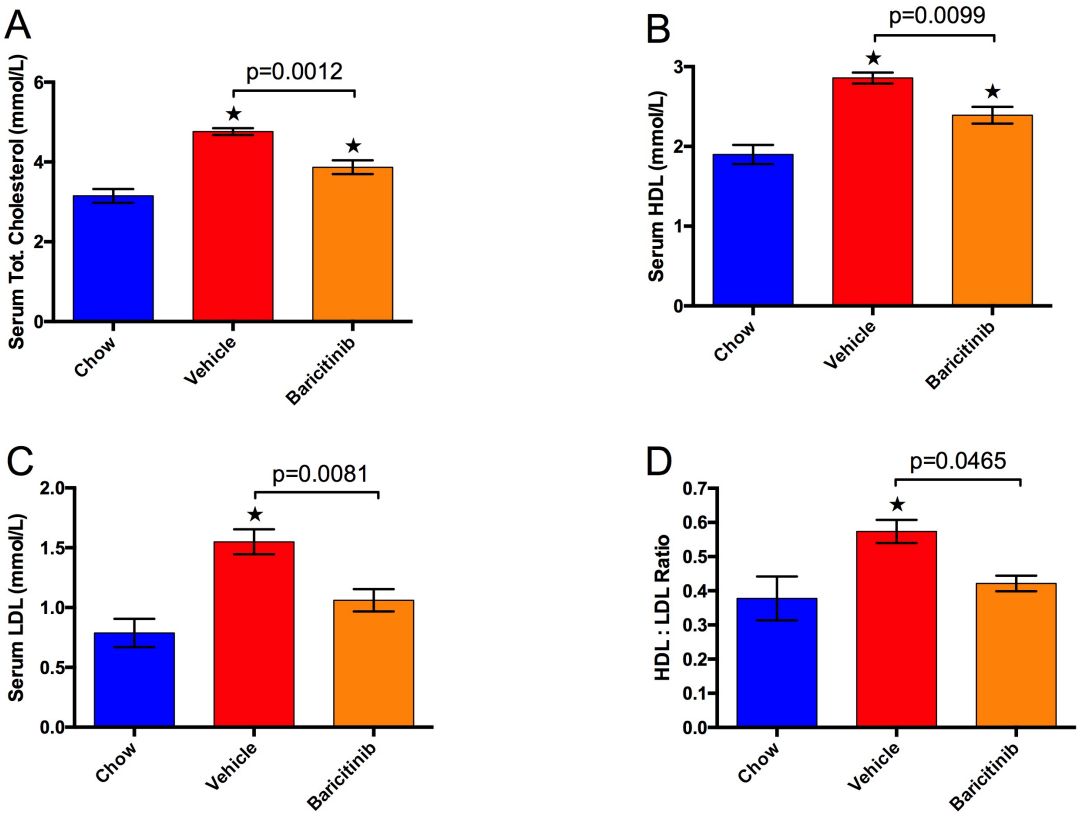

Figure 4.6. Serum lipid measurements for animals fed with HFD and treated with vehicle or baricitinib 10mg/kg compared with mice fed chow diet. A= total cholesterol. B= HDL. C= LDL. D= HDL: LDL ratio. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Serum Cytokines

Serum KC (figure 4.7.A) was significantly reduced in baricitinib animals against vehicle but no significance against chow. Neither vehicle nor baricitinib groups showed significance against chow in spite of a mild increase in the vehicle group against chow. Serum TNFα (figure 4.7.B) was decreased in baricitinib animals versus chow and vehicle with no significance between chow and vehicle. IL-2 (figure 4.7.C) was significantly elevated in the baricitinib group versus vehicle but not against chow with no significance between chow and vehicle. IL-10 in the serum (figure 4.7.D) was significantly elevated in the vehicle group against both chow and baricitinib with no significance between chow and baricitinib. Serum IL-6 (figure 4.7.E) and IFNγ (figure 4.7.F) showed no significant changes in any groups in spite of a mild decrease in the baricitinib group for both.

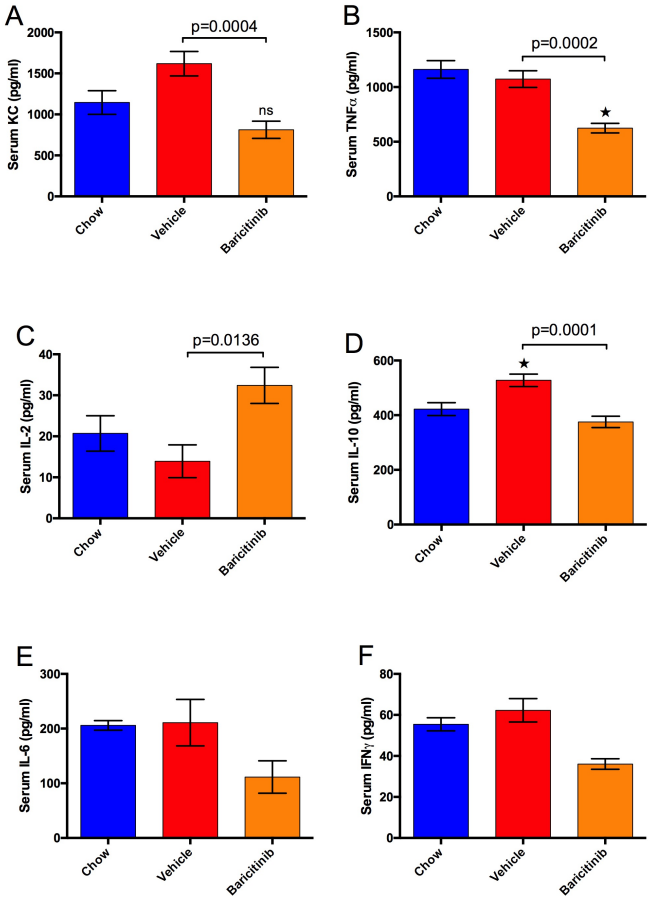

Figure 4.7. Data for cytokines measured from serum of animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=8. Baricitinib: n=8. Data presented as mean ± SEM.

Serum Hormones/Incretins

Serum insulin (figure 4.8.A) was significantly elevated in vehicle and baricitinib groups against chow, with no significant differences found between vehicle and baricitinib. Serum glucagon (figure 4.8.B) is significantly higher in baricitinib animals versus both chow and vehicle groups, which showed no significance between each other. Serum GLP-1 (figure 4.8.C) is significantly decreased in baricitinib treated animals versus chow and vehicle, chow and vehicle groups showed no significance between them.

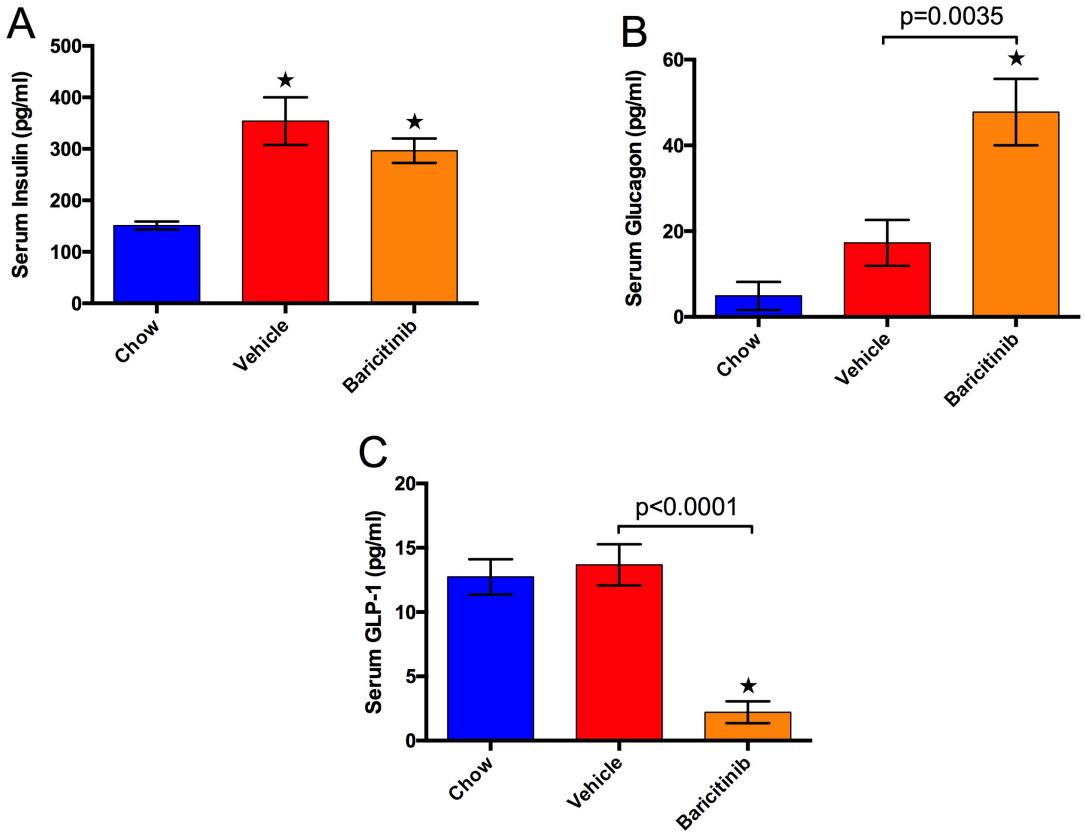

Figure 4.8. Measurements made of serum levels of the glucose controlling hormones insulin (A) and glucagon (B) along with those of the gut incretin, GLP-1 (C) for animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=8. Baricitinib: n=8. Data presented as mean ± SEM.

Serum Albumin

Serum albumin was found to be significantly decreased in baricitinib animals versus both vehicle and chow mice. No significance was shown between vehicle and chow mice.

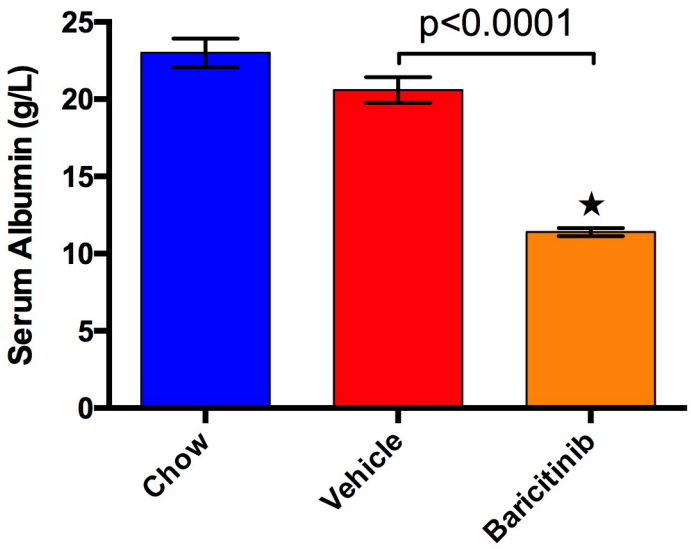

Figure 4.9. Data for serum albumin measurements from animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=8. Baricitinib: n=8. Data presented as mean ± SEM.

The Diabetic Condition

Blood Glucose

Random blood glucose measurements from the end of the study show that there is a significant increase in the blood glucose of vehicle mice against chow. A decrease in this was observed in baricitinib mice versus vehicle, but which was just non-significant, baricitinib remained non-significant against chow.

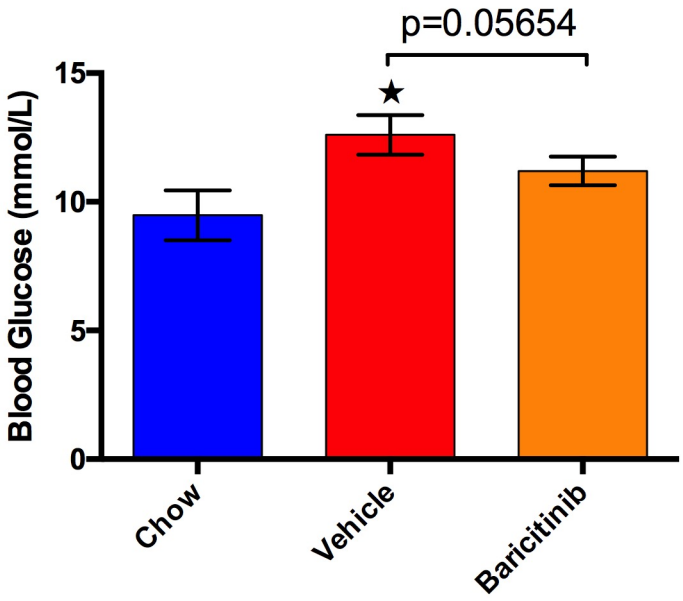

Figure 4.10. Data for a random blood glucose measurement at termination of animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Food Intake

Food intake in grams (figure 4.11.A) was found to be significantly lower in animals in the vehicle and baricitinib groups versus chow fed animals throughout. For the most part there was no significance between vehicle and baricitinib animals, apart from week 7 where food intake for animals in the baricitinib group sharply declined followed by a recovery in week 8. AUC analysis of the data in figure 4.11.A (figure 4.11.C) confirmed that there was a lowering in the amount of food eaten by mice in vehicle and baricitinib groups but that it was only significant against chow in the baricitinib group. Calorific intake (figure 4.11.B) was found to be significantly higher in vehicle and baricitinib groups versus chow at a number of points in the study, when this was not significant it was still above the chow level throughout. AUC analysis of the data in figure 4.11.B (figure 4.11.D) did not reveal any significance but did show a small, non-significant, increase in both vehicle and baricitinib groups over the chow group.

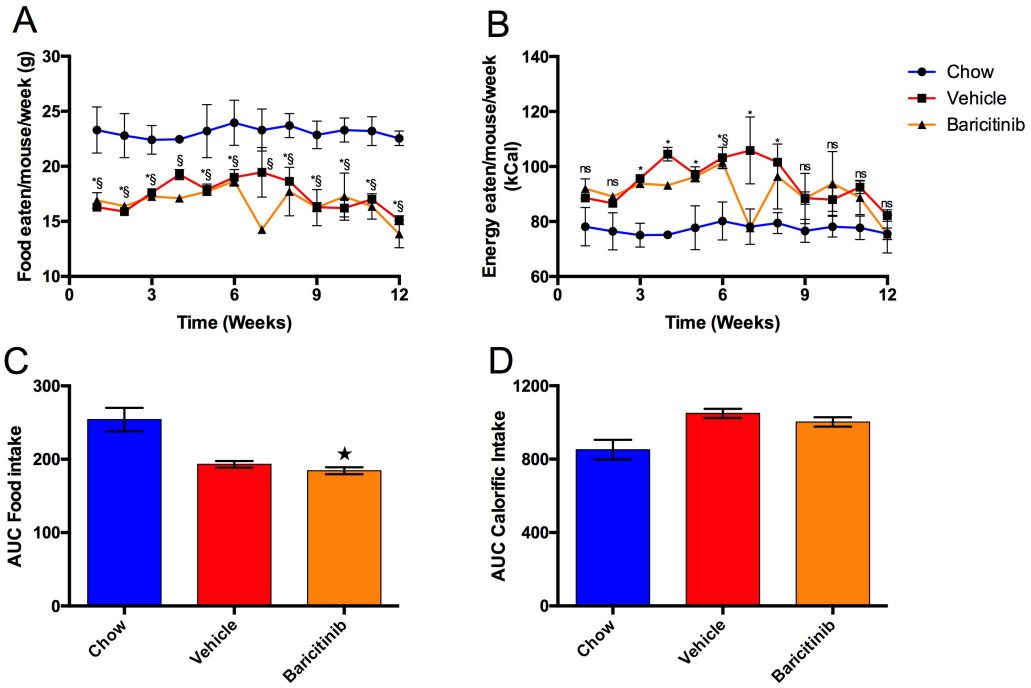

Figure 4.11. Food (A) and energy (B) intake data and analysis of animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. Also, AUC analysis data for food (C) and energy (D) intake. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. * P≤0.05 baricitinib vs. chow when analysed by two-way RM-ANOVA. § P≤0.05 vehicle vs. chow when analysed by two-way RM-ANOVA. 'Biological n': Chow: n=9. Vehicle: n=10. Baricitinib: n=10. 'Datapoint n': Chow: n=2 cages. Vehicle: n=2 cages. Baricitinib: n=2 cages. Data presented as mean ± SEM.

Weight Gain

Vehicle and baricitinib groups show an enhanced rate of weight gain versus chow in figure 4.12.A with this difference becoming significant from week 4 onwards, no significance was found between vehicle and baricitinib at any point. All groups gained weight throughout the study except vehicle and baricitinib groups at week 8 where they lose weight briefly before gaining again in week 9. AUC analysis (figure 4.12.B) showed that the overall difference in weight gain was significantly higher in the vehicle and baricitinib groups versus chow with no difference between vehicle and baricitinib.

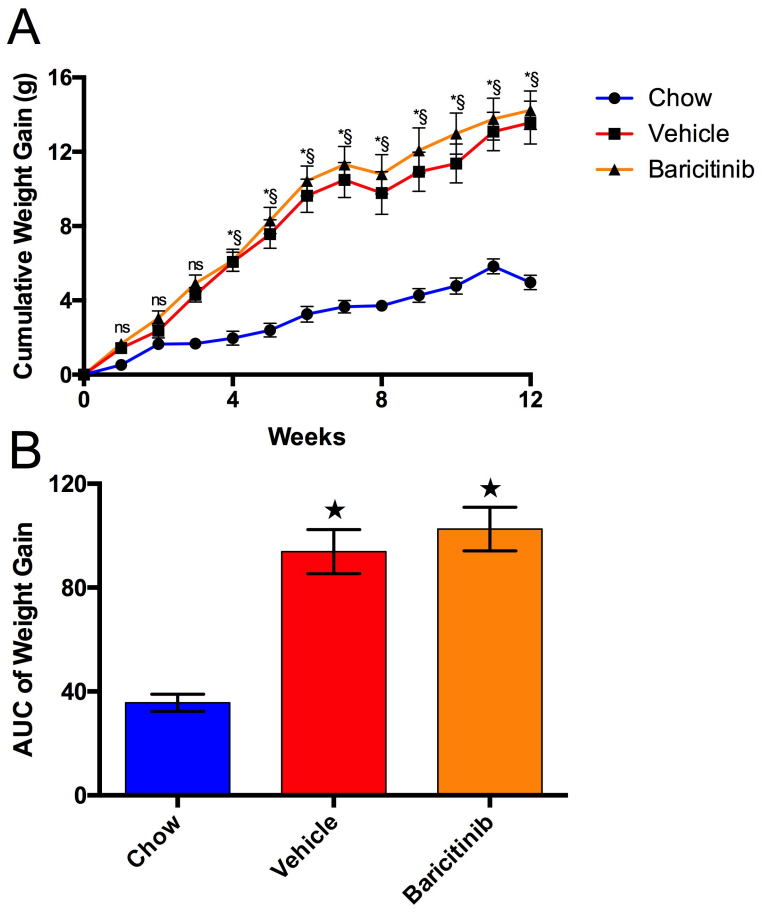

Figure 4.12. Weight progression over the 12-week HFD study for animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. * P≤0.05 vehicle vs. chow when analysed by two-way RM-ANOVA. § P≤0.05 baricitinib vs. chow when analysed by two-way RM-ANOVA. Chow: n=8. Vehicle: n=9. Baricitinib: n=10. Data presented as mean ± SEM.

Magnetic Resonance Imaging

Representative MRI images of mouse quadriceps muscles analysed are shown in figure 4.13, areas in red show ROI for the quadriceps muscle and areas in green display areas exceeding the fat threshold. Figure 4.13.A shows the raw image of chow mouse quadriceps muscle and 4.13.D shows the amount of fat present in chow mice muscles in green. Figure 4.13.B shows the raw image of vehicle mouse quadriceps muscle and figure 4.13.E displays the amount of fat present in vehicle mice muscles in green. Figure 4.13.C shows the raw image of baricitinib mouse quadriceps muscle and figure 4.13.F displays the amount of fat present in baricitinib mice muscles in green.

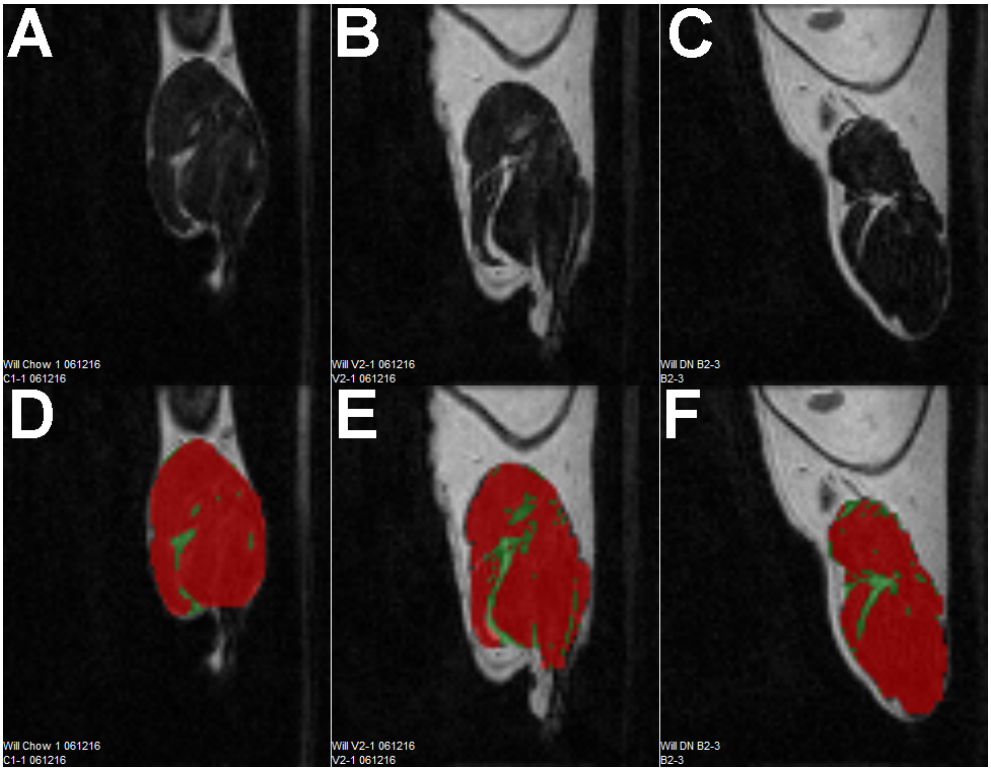

Figure 4.13. Representative MRI images with analysis from mice fed with chow (A&D) or HFD and treated with vehicle (B&E) or HFD and treated with baricitinib (C&F).

Analysis of the data from MRI scans is displayed in figure 4.14, this data relates to the images shown in figure 4.13. Quadriceps muscles in animals in the vehicle and baricitinib groups show a significant increase in their proportional fat content compared to chow animals. Baricitinib animals displayed a significantly lower level of fat compared to vehicle but this was still significantly higher than that seen in chow animals.

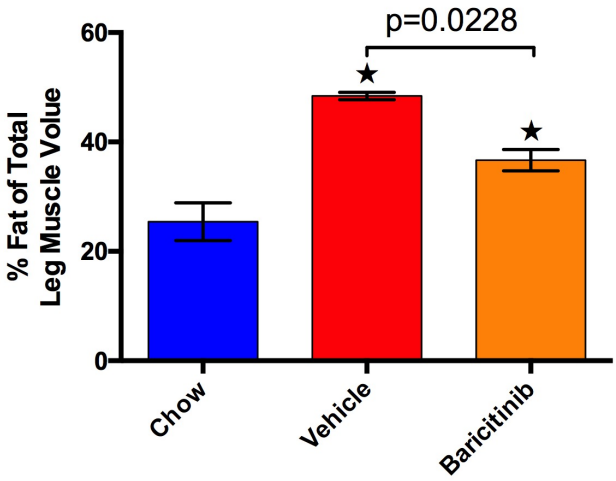

Figure 4.14. Representative images and volumetric analysis of perimuscular fat in the quadriceps muscles of animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. Chow: n=4. Vehicle: n=4. Baricitinib: n=4. Data presented as mean ± SEM.

Tissue Analysis

Adipose Cytokines

IL-10 (figure 4.15.A) was significantly elevated in baricitinib mice versus both chow and vehicle mice with no change between chow and vehicle groups. IL-1β (figure 4.15.B) was significantly elevated in vehicle mice versus both chow and baricitinib with no significance between chow and baricitinib. IL-6 (figure 4.15.C) was significantly higher in vehicle mice versus chow, with no significant change between baricitinib and chow or baricitinib and vehicle. KC (figure 4.15.D) was found to be significantly higher in vehicle and baricitinib mice versus chow, baricitinib mice were significantly lower than vehicle but still elevated versus chow. TNFα (figure 4.15.E) was significantly decreased in baricitinib mice versus chow and vehicle with no significance between chow and vehicle.

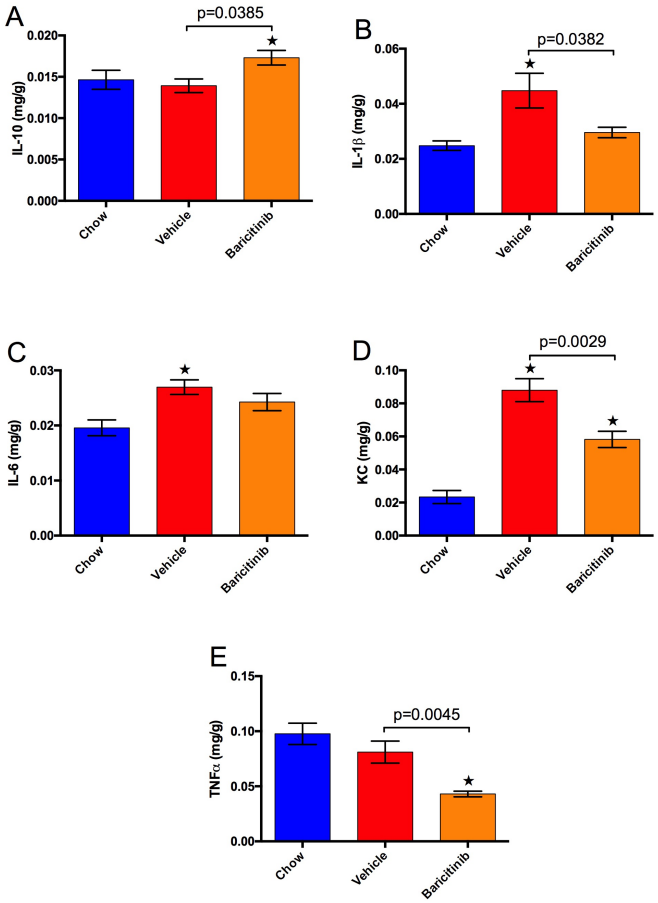

Figure 4.15. Cytokine analysis for adipose tissue from animals fed HFD and treated with vehicle or baricitinib (10mg/kg) or those fed chow diet. ★ P≤0.05 vs. chow when analysed by one-way ANOVA. Chow: n=8. Vehicle: n=8. Baricitinib: n=8. Data presented as mean ± SEM.

Liver Oil-Red-O Staining

Below in figure 4.15 are representative images of oil-red-O staining carried out on sections of liver from HFD and chow animals, this data could not be quantified due to contamination of the samples which is seen as black spots on the below images. It appears from qualitative analysis of these sections that there is a marked increase in hepatic fat deposition with HFD feeding. There also appears to be an increase in the size and quantity of oil-red-O positive staining in the baricitinib livers versus those from the vehicle treated group.

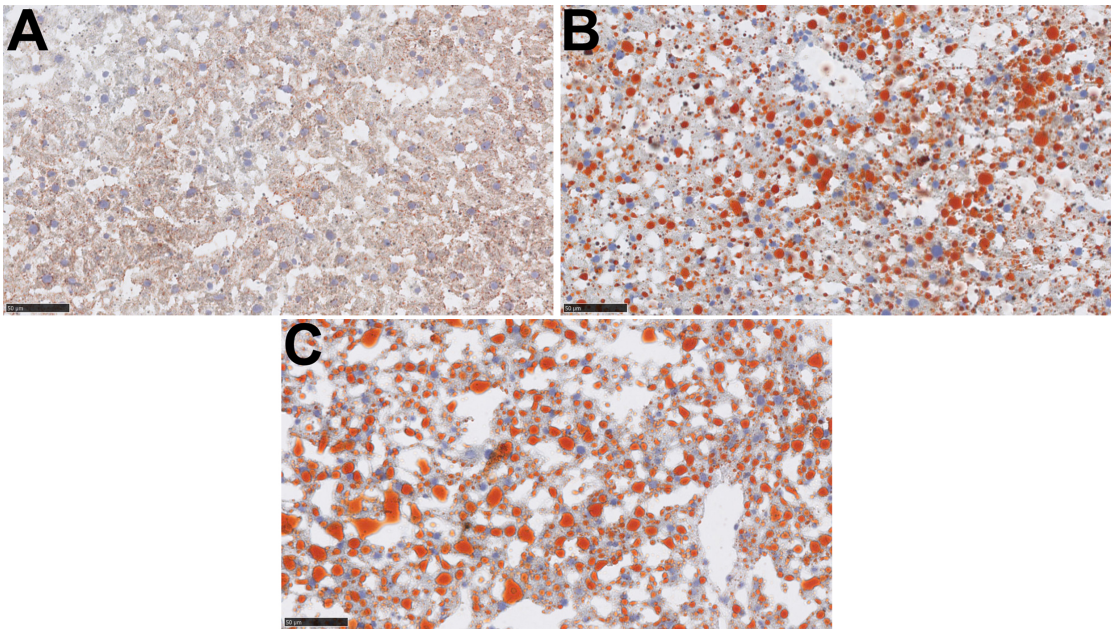

Figure 4.15. Representative sections of liver from animals fed HFD and treated with vehicle (B) or baricitinib (10mg/kg) (C) or those fed chow diet (A) stained with oil-red-O stain for lipids.

Discussion

The most striking result of these investigations was in fact an accidental discovery. The reduction in high-fat induced cholesterol is surprising and opens up new questions. A drug that reduces hypercholesterolaemia to basal levels is an extremely powerful tool, especially when this drug is potentially displaying positive effects on other aspects of a disease. This discovery warrants further investigation to elucidate the mechanism by which it is mediating these effects and potential side effects.

Given the suggestions of Khan et al., (2015) that baricitinib plays a role in the modification of the actual diabetes disease state by reducing insulin resistance it would be logical to think that in this case, the disease is less severe therefore this justifies the change in lipid levels. None of the evidence presented here in this thesis suggests that baricitinib has in anyway improved the disease state of these animals. The random glucose measurements show that glucose is still elevated against chow, albeit not significantly. This paired with the data from OGTT and ITT, presented in chapter III show that there is no improvement in insulin sensitivity because of baricitinib treatment.

The next most impressive change in these animals' physiology made by baricitinib is that of the abolition of GLP-1 signalling in the serum. GLP-1 has little direct effect on glucose metabolism rather mediating their effects via modification of pancreatic α and β-cells (Baggio & Drucker, 2007). So, the effects of this vast reduction in GLP-1 in the serum are noticeable in the modulation of insulin and glucagon. Our result show the expected changes in these hormones associated with drops in GLP-1 levels; reduced (mildly) insulin and increased (significantly) glucagon. Glucagon has before been implicated in controlling the rate of cholesterol synthesis by inhibiting the rate limiting cholesterol-synthesising enzyme, HMG-CoA (Cook et al., 1977, Ingebritsen et al., 1979). It also stands to reason that glucagon

is going to increase the uptake and metabolism of all fats, withdrawing them from the circulation (Aydin & Sokal, 1963). Indeed, recent studies have shown that incubating hepatocytes with glucagon reduces the activity of SREBP genes (Horton, Goldstein & Brown, 2002) which are involved in cholesterol synthesis, meaning that there is less production of cholesterol in the liver and so lower circulating cholesterol. The most recent studies in this area show that there is a direct effect of hypergulcagonaemia on lowering hepatic generation of VLDL and LDL in humans, proving the cholesterol lowering effects of acute glucagon increases in lowering serum cholesterol (Changting et al., 2011). This study showed that in humans subjected to a 3-fold increase in glucagon had a significant reduction in LD. In our study the effect is more profound but as is our increase in glucagon being around 10-times basal levels. Our study suggests that the effects of hyperglucagonaemia carry through to a chronic setting and can potentially be re-created pharmacologically.

The changes in GLP-1 do not seem to influence the diabetic condition; the very mild reduction in insulin levels is not significant against vehicle and remains significant against chow fed mice. Blood glucose remains non-significant in its changes between vehicle and baricitinib groups and hunger is unchanged between HDF groups, the changes observed in insulin are non-significant and are sub-clinical, there may be a case to be made for this becoming significant in a longer-term study so this warrants further investigation.

Another justification for the lowered serum cholesterol may be found in the fat itself as the source of fatty acids. Inflammation within adipose tissue has for a long time been acknowledged as a causative factor in diabetes and metabolic syndrome (de Ferranti & Mozaffarian, 2008). Our results show that there is a decrease in the markers of inflammation in adipose tissue in animals treated with baricitinib versus vehicle treated animals. Animals fed HFD show an increase in key cytokines versus chow control, this is thought to result in an

increase in serum lipids (Van Gaal, Mertens & Christophe, 2006). Adipose inflammation is thought to lead to fat-remodelling and deposition of lipid in muscles (Khan et al., 2015) and vascular tissues (O'Keefe & Bell, 2007) leading to complications like CVD and increased insulin resistance. Our findings show that there is a decrease in not only the inflammation of the adipose tissue but also the downstream effects of lipid deposition, shown by reduced perimuscular and intramuscular fat deposits by MRI analysis.

It is particularly noticeable that IL-6 in the serum is reduced by administration of baricitinib. IL-6 is thought to be one of the key mediators of diabetic adipose inflammation (Sultan et al., 2009), but is not significantly reduced in adipose tissue with baricitinib treatment. Baricitinib must be causing this anti-IL-6 effect somewhere other than in the fat.

Interleukin-6 signalling results in an increase in the production of GLP-1 (Ellingsgaard et al., 2011) so a decrease in IL-6 would logically reduce GLP-1 production from intestinal L-cells. However, there may be another mechanism at play related to the IL-6/GLP-1 axis, the IL-6 receptor associates with Jak/STAT proteins (Stahl et al., 1994) in order to induce changes in its target cells. The blockade of the Jak/STAT pathway in IL-6 signalling would mean that IL-6 mediated GLP-1 release would be inhibited. Our results show that there is an enormous activation of the Jak/STAT pathway in conditions that are heavily linked to IL-6; this is shown by the upregulation of Jak/STAT associated genes in LPS challenged mice. LPS administration models the human condition sepsis, which is known to have a strong link to IL-6 release (Riedemann et al., 2003).

It could be that what is happening here is, rather than a return to normal signalling, or abrogation of adipose inflammation, Jak/STAT inhibition is inducing physiological changes that bring about a reversal of the symptom. Baricitinib is not treating the root cause of

hyperlipidaemia, which is poorly understood and may be as a result of oxidative stress in the adipose tissue (Baynes, 1991), but in fact activating an alternative mechanism to lower the levels of cholesterol in these animals.

The ultimate cause of the reduction in cholesterol observed in this model may well be multifactorial. The initial levels of fat circulation reduction could be as a result of decreases in inflammation in the adipose tissue caused by the administration of baricitinib. However, the specific decreases in cholesterol cannot be explained simply by this, and the absence of modulation of circulating triglycerides casts doubt on this conclusion. It means that there must be another mechanism at play, reducing the levels of cholesterol in the serum of animals treated with baricitinib, this mechanism, I suggest is that of IL-6 signalling blockade resulting in depletion of GLP-1.

These results should be treated with caution as alterations of liver metabolism can be as a result of hepatotoxicity. Our results see that there is an apparent increase in the hepatic storage of lipid, which is associated with cirrhosis (Farrell & Larter, 2006) and there is also a reduction in serum albumin, which is again, potentially indicative of liver damage (Reilly et al., 1990). It should also be highlighted that previous Jak inhibitors have been found to induce hyperlipidaemia in patients taking them for RA (Kontzias et al., 2012) so these findings are contrary to those previously published in humans. Any studies in humans however have been carried out using physiological doses of around 2-4mg QD, assuming a human of 80kg that amounts to a dose of 25-50μg/kg compared to the dose in this study of 10mg/kg.

Chapter V: General Discussion and Conclusions

These studies have covered a large scope of pharmacology, basic science and translational medicine making it a tough task to unite all the findings into one coherent, uniting theory. It has long been thought that there is a link between the clinical conditions of diabetes and natural ageing (Mbanya, 2012) and they constitute a heavy burden on healthcare systems. There is currently no 'magic bullet' treatment for diabetes and, therefore, patients with diabetes are often some of the most heavily medicated patients in hospital and community care (Peron, Ogbonna & Donohoe, 2015). These drugs are leading to a population of diabetic patients that are living increasingly long lives, combining their diabetic condition with the complications of natural ageing, leading to polypharmacy in these patients (Good, 2002). With every new drug that is added to an ageing patient the risk of drug-drug interactions and negative reactions increases (Gorard, 2006). There is now a call for treatments aimed towards treating the condition or at least ones that will have a positive effect on many of the linked pathologies. This thesis has attempted to assess the possibility that baricitinib is a step closer to finding a drug that can treat multiple pathologies associated with DM.

The first conclusion to make is that the oral DGal model of advancing age, is indeed not a model of advanced age at all. This model has been shown here to not be linked to ageing in the general viscera but in the brain alone. Though the model has been used previously to stimulate ageing in the renal tissues (Aydin et al., 2012), our model showed that these results may be flawed. It could be that the difference in route of administration (oral versus i.p.) resulted in a lower plasma concentration of DGal that was high enough to result in changes in the brain but not in the kidney. It may also be that organs such as the kidney and the liver, where we studied, are more resistant to this insult than organs like the brain, so injury was still caused but at a level below that which we could measure. Finally, it could be argued that we did not dose these animals for long enough for the effect to be seen in the peripheral circulation of these animals. The brains of these animals were harvested for

another project in the CNS division of Takeda Cambridge Ltd., so they were injured suitably in the brain but not in the kidneys, livers, lungs and other organs which we studied. The fact that brain injury was observed in these animals but not organ damage in other organs leads me to conclude that the DGal model of advanced age is not suitable to study ageing in mice outside of the brain.

Our investigations into baricitinib have shown clearly one thing above all, and that is that baricitinib is not a drug that can improve insulin sensitivity and 'roll back the clock' on diabetes. We should not see this drug as the 'magic bullet' as it has no effect on the diabetic condition itself under our conditions. Hyperglycaemia is still the most prominent symptom of diabetes and needs to be treated at the earliest stages of the disease where possible, drugs like the anti-hyperglycaemic metformin and the DPP-4 inhibitor sitagliptin continue to show clinical efficacy and remain the first line treatments for diabetes for good reason (Aschner et al., 2006, Klepser & Kelly, 1997, NICE 2016).

DN is currently treated with angiotensin-converting enzyme (ACE) inhibitors for the underlying hypertension that can lead to DN and worsen it. This method of treatment focuses on prevention of the disease progression and protection of existing basement membrane integrity (Lewis et al., 1993). The findings of Tuttle et al., (2015) suggested that Baricitinib could lead to a recovery of renal function in humans with DN; my work supports this. My results indicate that baricitinib plays a role in the protection of the glomerulus from mesangial expansion and podocyte loss.

My findings regarding baricitinib are like those found relating to the anti-diabetic drug troglitazone which was found to prevent mesangial expansion (McCarthy et al., 2000). troglitazone is an anti-diabetic drug of the class glitazones (thiazolidinediones), it mediates

its effects by acting as a ligand the peroxisome proliferator activated receptor-γ (PPARγ). PPARγ is one of three nuclear sensors of fatty acid concentration which act as transcriptional activators, the others being, PPARα and PPARδ. Glitazones act to increase insulin sensitivity in multiple tissue types; these include adipose, liver and skeletal muscle. In muscle tissue, uptake of glucose is promoted by increased PPARγ activity resulting in higher expression of GLUT-1 allowing increased flux of glucose into the cell (Zierath et al., 1998). Increased glucose in myocytes is followed by heightened activity of Glucose-6-Phosphate (G6P) and higher rates of glycogen synthesis (Petersen et al., 2000). In adipose a similar increase in not only GLUT-1, but also GLUT-4 (Sandouk, Reda & Hofmann, 1993) is observed under the action of glitazones followed by a decrease in lipolysis and promotion of lipogenesis (Gastaldelli et al., 2009). Finally, in hepatocytes, GLUT-2 is upregulated under application of glitazones (Smith, 2002) with decreased glycogenolysis, one of the key actions of the liver under insulin stimulation (Miyazaki et al., 2001).

Glitazones have a positive effect, as might be expected given their mode of action, on glucose tolerance (DeFronzo et al., 2011) and insulin tolerance (Nolan et al., 1994). These compounds not only have a sensitising effect on various tissues but also increase insulin release by sensitising pancreatic cells to glucose, by increasing GLUT2 expression. Below in figure 5.1, can be seen a graphic representation of the mode of action of glitazones in the liver and pancreas by activating PPARγ.

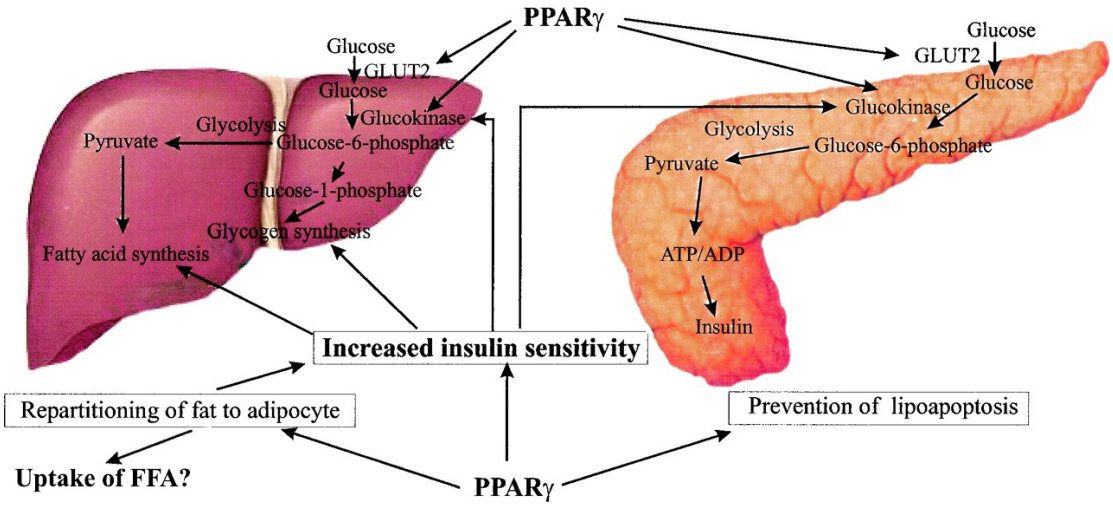

Figure 5.1. Graphic representation of PPARγ action on glucose sensitivity, from top and insulin sensitivity, from bottom (adapted from Kim & Ahn, 2004).

An additional feature of glitazone administration is a reduction in LDL cholesterol and serum lipids (Gastaldelli et al., 2009). The putative mechanism for this is that there is a reduced 'flux' of lipids from adipose tissue to liver and an associated decrease in ApoB production meaning that lipoproteins become increasingly dense (Seedorf & Aberle, 2007). Figure 5.2 shows the effects of PPAR's on the flux lipids between adipose and hepatic tissues, where we see a role for PPARγ in preventing the release of FFA's into the circulation and to the liver. Other PPAR's, PPARα and PPARδ show activity in preventing ApoB expression, meaning that there is a lower release of LDL and VLDL from the liver.

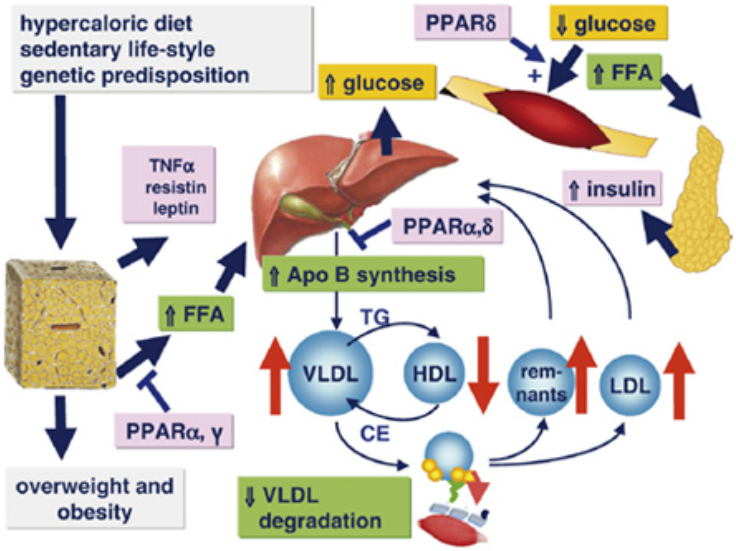

Figure 5.2. Lipid flux in obesity and HFD with a central role for PPAR's (adapted from Seedorf & Aberle, 2007).

Glitazones have been shown to interact with a number of inflammatory and cell cycle regulatory proteins (Chinetti, Fruchart & Staels, 2000). There is currently only one glitazone available on the market, pioglitazone, there have been a number of other glitazones, such as troglitazone and rosiglitazone, which were withdrawn due to hepato-toxicity. It is suggested that the interactions of PPARγ agonists promoted necrotic and apoptotic processed in hepatocytes leading to liver damage (Guo et al., 2006).

The work of McCarthy et al., (2000) suggests that the activation of PPARγ prevents mesangial expansion due to increased glycaemic control. Their results, in terms of reductions in mesangial expansion and urinary ACR were like those presented here. Our results go deeper than theirs into the mechanistic background of our drug. Given the fact that baricitinib does not have any effect on insulin or glucose sensitivity and yet there is an improvement in mesangial expansion, it seems unlikely that the reduced mesangial expansion is due to increased glycaemic control.

The overall picture of baricitinib in HFD and diabetes looks rather similar to that of many glitazones, like troglitazone. Lowering of serum lipids, especially cholesterol and preventing mesangial expansion are found both with some glitazones and baricitinib in our experiments. However, there is a glaring difference in the effects of these two drugs, our results find that there is no difference in insulin or glucose sensitivity between animals on baricitinib and those on vehicle treatment whereas glitazones have a proven effect on this.

There is a proposed mechanism of Jak2/STAT3 inhibition by PPARγ similar to that of SOCS3 (Yu, Kim & Kim, 2008). The work of Yu, Kim & Kim (2008) shows that PPARγ activation not only increases SOCS3 expression which has long been known to inhibit Jak/STAT activation but also that it directly inhibits STAT3. Figure 5.3.A shows the process of negative feedback

by SOCS on cytokine induced STAT translocation and transcription, whereby unphosphorylated Jak is targeted for proteosomal degradation. Similar effects of PPARγ have been found and a representation of this is seen in figure 5.3.B where STAT translocation is prevented by inhibition of both Jak and STAT by activated PPARγ.

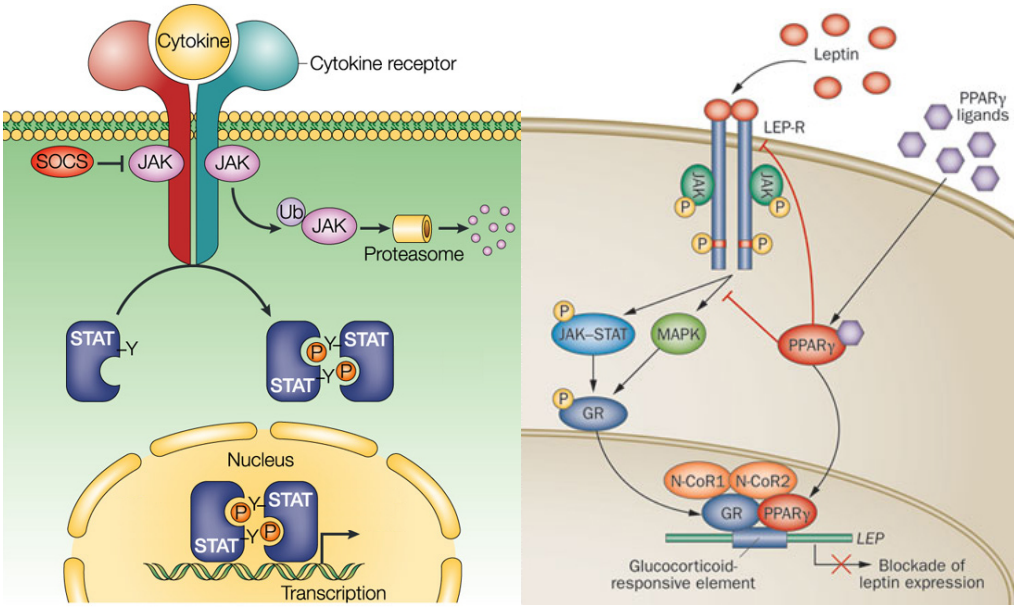

Figure 5.3. A, SOCS inhibition of JAK/STAT signalling that results in proteasomal degradation of Jak and abrogation of Jak/STAT Signalling (adapted from Andò & Catalano, 2012). B, a similar pathway to A where PPARγ inhibits Jak phosphorylation, is preventing STAT dimerisation and translocation (adapted from Shuai & Liu, 2003).

It has been discussed earlier in this thesis that inhibition of Jak phosphorylation is similar in action to increasing SOCS (Yoshimura, Naka & Kubo, 2007). Considering the action of PPARγ when activated by ligands, such as glitazones, it is feasible that the actions of glitazones are being replicated by baricitinib in our experiments. The findings of McCarthy et al., (2000) do not mention the induction of cell cycle arrest or induction of apoptosis but the work of Okada et al., (2006) specifically implicates pioglitazone in cell cycle arrest in amelioration of DN. Okada et al., (2006) specifically implicates increased $p21^{cip1/waf1}$ expression in this process, providing a very strong link between the mode of actions of baricitinib and pioglitazone. This is further supported by *in vitro* work by Vitale et al., (2012) whereby cell survival induced by STAT is antagonised by the action of PPARγ.

The similarities in the findings for using baricitinib in a HFD induced mouse model of DM and the known effects of glitazones lead me to postulate that there is a uniting factor. The antagonistic effects of activated PPARγ on Jak/STAT activation observed by Yu, Kim & Kim (2008) coupled with known effects of SOCS on Jak/STAT activation (Yoshimura, Naka & Kubo, 2007) lead to the conclusion that STAT acts antagonistically to PPARγ. In this thesis, it has been shown that Jak/STAT inhibition is associated with a reduction in glomerular hypertrophy similar to that shown by glitazones therapy.

The lack of insulin sensitisation in mice treated with baricitinib leads me to suggest the theory that STAT antagonises the action of activated PPARγ with respect to lipid metabolism and glomerular hypertrophy but not in glucose metabolism. Evidence that links STAT to glucose metabolism has been found but it further provides a strong link to PPARγ (Inoue et al., 2004). It may be the case that in some situations, there is an increase in both glucose and insulin sensitivity with reductions in Jak/STAT signalling but our results have not shown this and therefore do not support the theory that baricitinib is acting via the PPARγ pathway. Perhaps it is possible that the effects are being replicated in tissues other than the pancreas where the Jak/STAT machinery is more closely linked to PPARγ and that the SOCS-like effects of PPARγ are not present in the pancreas under normal or diseased conditions.

The profile of animals on HFD treated with baricitinib is strikingly similar to that that one would expect of one treated with a glitazone. Our mice lacked sensitisation to insulin or glucose which would be expected of animals on a glitazone, this leads us to believe that Jak/STAT inhibition results in a lowering of lipid profiles and reduction in mesangial expansion by a similar mechanism to that of PPARγ activation but that it has a sub-clinical effect on glucose metabolism. Our results are not conclusive on whether there is a direct mechanistic link between STAT and PPARγ activation but current literature would suggest

that there is a possibility of a direct link between the two, certainly warranting further investigation.

Ageing in These Investigations

It is important to consider the effects of all drugs on those with advanced age, as was the aim of these investigations from the outset. As has already been mentioned, elderly diabetic patients present an at risk group for polypharmacy and the associated dangers of that. The conclusions of this research suggest a possible link between PPARγ activators and STAT inhibitors, both of these classes of drug have members that are approved by both the FDA and EMA for treatment of DM and RA, respectively. The main concern with elderly patients on any chronic treatment is the risk of adverse events and very few drugs are without the potential of dangerous interactions with other drugs.

There is currently one FDA approved PPARγ agonists on the market after troglitazone was withdrawn in 2000 due to safety concerns over cardiac and hepatic affects. Pioglitazone is used commonly in combination with metformin for the treatment of diabetes (NICE, 2016) with other PPAR agonists in development for obesity and dyslipidaemia. There are still concerns over the safety of PPARγ activators regarding their safety (Balakumar & Mahadevan, 2012, Salomone, 2011) however studies have shown they have no increased risk in elderly patients (Rajagopalan et al., 2004).

Baricitinib is under review by the FDA as of January 2017 and approved for use by the EMA as of December 2016 and safety studies are indicating that it is well tolerated at therapeutic doses for RA (Keystone et al., 2015). The drug is a relatively new drug and so widespread use of the drug is not common meaning that the population exposed to the potential for adverse events is small. Current adverse events include neutropenia and hyperlipidaemia (Shi et al.,

2014) though it has been discussed earlier in this thesis that the doses used in current clinical trials are vastly lower than those used in our experiments. In terms of the reaction of the elderly to baricitinib, there is scant data however Fleischmann et al., (2016) present a case that overall adverse events are more common in the elderly with no increase in serious events.

Given the suggestion that PPARγ agonists and STAT inhibitors in this setting result in similar effects that may be mechanistically linked it would be important to keep in mind the possibility that baricitinib, at doses similar to those presented here, would result in the same adverse effects as seen with glitazones. It seems that there is very little difference in the pharmacological features of these drugs with baricitinib (Shi et al., 2014) and pioglitazone (Deng, Wang & Li, 2005) both having half lives in the region of 6-9 hours.

Baricitinib appears not to represent a 'magic bullet' drug that induces positive effects on all aspects of diabetes. It has similar effects to that of glitazones drugs that are already used in the treatment of diabetes but does not modify the disease itself. It is hard to envision the use of a drug that has the effects of baricitinib being used preferentially over a drug that also improved insulin sensitivity, all things being equal. However, all things in this study were not equal, dosage and head-to-head comparisons need to be carried out before we can say that there is a preference either way. Baricitinib, as we see it now, has a preferable safety profile over glitazones so it would be prudent to examine the possibility of this drug being a safer alternative to glitazones with additional use of other drugs to combat the insulin sensitivity.

Inflammation related to ageing may well have a part to play in the damage to nutrient sensing in ageing and therefore potential predisposition to diabetes. Baricitinib showed scant anti-inflammatory properties, except in TNFα and had little effect on the key ageing

related cytokines, KC and IL-1β. The effects of baricitinib do not match up with the proinflammatory ageing phenotype that was found in chapter II. This lack of anti-inflammatory action means that baricitinib would likely have little effect on inflammaging, but its organ protective effects could still be mediated by other means and could warrant further exploration.

The effects of ageing on renal anatomy, including glomerular hypertrophy and capsule expansion shown in chapter II are similar to that shown in chapter III. This could contribute to the worsening of DN in elderly patients and given the similarity in morphology could be as a result of mesangial expansion. Elderly patients with DN on a glitazone (for diabetes) or Jakinib (for RA) may be having their disease severity reduced as a side effect. It could be that elderly patients with sub-clinical nephropathies already on either of these classes of drugs are not presenting with the clinical syndrome due to the inhibitory effects of these drugs on DN. Both PPARγ activators and jakinibs could prove a useful clinical drug in treating the effects of diabetes and as a prophylactic drug for nephropathy. This could be particularly applicable for elderly patients on long-term treatment for DM or RA meaning that they are being protected against the pathological aspects of renal ageing simply be being on these drugs.

Baricitinib is not going to be the 'elixir of life', keeping patients forever young. But given its beneficial effects of the metabolic syndrome of HFD-induced DM and on DN it could have far-reaching positive effects on elderly patients.

Conclusions

- Elderly mice have a pathology, independent of exterior factors which includes:-
    - Insipid metabolic syndrome characterised by:-
        - Reduced insulin sensitivity
        - Reduced glucose sensitivity
        - Inappropriate insulin release
    - Sub-clinical histological changes in renal structure such as:-
        - Glomerular hypertrophy
        - Bowman's capsule expansion
    - Whole-body inflammation particularly:-
        - Increased circulating activated immune cells
        - Increased tissue and circulating KC along with changes throughout the body in levels of IL-1β and other key cytokines.
- Baricitinib reduces experimentally induced diabetic nephropathy tentatively proposed to be as a result of:-
    - Prevention of glomerular hypertrophy
    - Changes in $p21^{cip1/waf1}$ action in renal cells
- Baricitinib reduces experimentally induced diabetic hyperlipidaemia tentatively proposed to be as a result of:-
    - Reduced circulating IL-6
    - Vastly reduced GLP-1
- Diabetes and natural ageing show similar pathological changes meaning:-
    - Diabetes can be more severe in advanced age
    - Treatments for diabetes could potentially help prevent ageing related pathologies in the kidney and whole-body

Future Investigations

Looking forward investigations could be focused, as these investigations have, on two fronts, that of the mechanical efficacy of baricitinib in DN and the lipid lowering effects of this drug in DN. Firstly, baricitinib's effects in DN have already been shown in human trials so its ability to attenuate diabetes-induced renal injury is not in doubt. Work that could help confirm the results and conclusions presented here in this thesis should include cell culture experiments relating to mesangial cells and the effect of multiple cytokine stimulation and blockade with baricitinib. Additional work on this aspect of the efficacy of baricitinib should include different models of DN induction including the streptozocin induced T1DM model.

One option in humans and animals that has not yet been explored in terms of DN is a head-to-head trial of other drugs with a similar mode of action. The first Jakinib to market was the oral Jak1/3 inhibitor, tofacitinib from Pfizer (Garber, 2013), this drug would likely be expected to have similar effects to that of baricitinib given similarities of the pathways they both inhibit and its proposed mode of action in RA. Other similar kinase inhibitors such as AG490 have been used in both *in vitro* (Wang et al., 2002) and *in vivo* (Banes et al., 2004) models of DN and have shown some interesting results similar to those seen in this thesis.

Clinical comparisons must be made using existing treatments for DN. Both pre-clinical and clinical head-to-head comparisons of baricitinib and ACE-inhibitors would potentially show the differences in mechanism by clinical effect. If baricitinib does in fact, as this thesis suggests, have a novel mode of action in terms of preventing and reducing diabetes-associated renal injury then there should be differences in the histology and signalling of the cells of the glomerulus in a head-to-head trial.

The final and potentially most important future extension to this programme of study to confirm the findings would be to attempt to replicate these results at a more physiological dose. The dosages used here are far in excess of any comparative dose that has been published in humans. A dose-response study would help to elucidate the finer mechanisms of this drug in DN.

The next phase of continuation for this study would be to evaluate the lipid-lowering effects of baricitinib and how it has come about in this model. Given the current evidence that baricitinib (Tanaka et al., 2016) and tofacitinib (Burmester et al., 2013) induce hypercholesterolaemia and data from phase II trials show a dose-dependent elevation in total cholesterol levels (Kremer, 2016), it is important that this result is developed further. The lipid-lowering effects of this drug could first be evaluated by administering it to animals on a chow diet to look for sub-normal depletion of cholesterol, as the proposed mechanism of this effect may be unrelated to the induction of diabetes. Also of interest would be the use of another model of hyperlipidaemia, such as a genetic model like that of the Watanabe heritable hyperlipidaemic rabbit (Kita et al., 1987). It should be considered that future investigations on this subject using animal models would use a different species also, as lipid metabolism varies between human, mouse and, for example, golden hamster (Liu and Wu, 2004), making the investigations less translatable. Time-progression evaluation of any future *in vivo* work on this drug in hyperlipidaemic animals would yield a great amount of information in terms of disease progression in these animals. *in vitro* work supporting this could be very useful indeed, to consider the mechanisms of IL-6 induction of GLP-1 signalling and blockade of this by baricitinib would confirm these findings.

Head-to-head trials of baricitinib and glitazones are essential for the conformation of the mimetic effects seen in this study. Control studies, using both drugs under identical conditions with comparable doses, is the only way to conclusively prove the link between PPARγ activation and Jak/STAT inhibition.

Both veins of further investigation would need to be tempered by the potential for hepatic impairment in these animals. Before any future human trials using this drug at equivalent doses to this study could be initiated proper and full pre-clinical evaluations of the hepatic implications of its use would need to be published. Pre-clinical evaluation of the drug showed efficacy using doses equivalent to ours (Fridman et al., 2010) and other studies (Khan et al., 2015) have also used 10mg/kg in mouse and rat studies with no reported adverse effects. Studies of RA (Fridman et al., 2010) only lasted 2wk meaning that there might not have been time to develop any hepato-impairment or this was not evaluated as it was not a primary outcome. Hepatic toxicology data has not been published or reported for this drug to date so the first step in evaluating the potential for clinical use of this drug at these doses would be to carry out full toxicology.

Without doubt the negative effects of baricitinib on the elderly are important to consider and further work based on Fleischmann et al., (2016) is needed with higher doses that appear to be therapeutic in DN. More than this though it is important that we look at the metabolic syndrome induced in natural ageing shown in chapter II and the potentially beneficial effects of baricitinib on this, given its effects in a model of HFD induced DN.

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